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# THE EFFECT OF TITANIUM SURFACE ROUGHNESS ON GROWTH, DIFFERENTIATION, AND PROTEIN SYNTHESIS OF CARTILAGE AND BONE CELLS

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**THESIS**

Presented to the Faculty of

The University of Texas Graduate School of Biomedical Sciences

at San Antonio

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Janet Yun-Chieh Martin, D.D.S.

San Antonio, Texas

May, 1996

THE EFFECT OF TITANIUM SURFACE ROUGHNESS ON GROWTH, DIFFERENTIATION, AND PROTEIN SYNTHESIS OF CARTILAGE AND BONE CELLS

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## DEDICATION

With great gratitude and relief, I dedicate this thesis to my husband, Michael William Martin, for his endless patience, love, and support throughout the entire periodontics residency program. I also dedicate this thesis to my parents for instilling in me the need to pursue educational excellence, and for their unfailing belief that there is nothing I cannot do.

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# THE EFFECT OF TITANIUM SURFACE ROUGHNESS ON GROWTH, DIFFERENTIATION, AND PROTEIN SYNTHESIS OF CARTILAGE AND BONE CELLS

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Placement of endosseous dental implants requires the growth and tight apposition of bone tissue to the implant surface. Histomorphological evaluation of various implant systems have shown variable contact between the bone and implant surface. Previous studies have alluded to the fact that various factors probably influence the interactions occurring at the bone-implant interface. These included specific surface characteristics such as chemical composition, surface texture and microgeometry, surface treatments including sterilization methods, presence of surface contaminants, and types of cells interacting at the implant interface. However, the role these factors play in osseointegration is not well known at this time. The object of this study, therefore, was to evaluate the effect of some of these factors on cell response.

Previous studies have used several different cell models to evaluate the effect of varying the nature of the implant. The possibility exists that each cell model may produce cell-specific results. Therefore, this study utilized 2 different cell models: a human osteoblast-like cell; the

MG63 cell line, as well as a well-characterized chondrocyte model, using primary cultures of the resting zone (RCs) and growth zone (GCs) chondrocytes, at two different stages of maturation. In this way, the effect of cell maturation on cell response could also be assessed.

A titanium substratum was chosen for these studies since most medical and dental implants are fabricated from titanium. The titanium was cut into uniform disks and then prepared in such a way as to produce surfaces of varying roughness and microgeometry. Steam autoclaving, a commonly used method of sterilization, was employed and the effect of multiple sterilizations was examined to determine if this process had any effect on either the characteristics of the surface or on cellular response.

The response of the cells to the varying surfaces was evaluated by assessing proliferation and differentiation, protein production and matrix synthesis. The biochemical assays used included the determination of cell number, [$^3$H]-thymidine incorporation as a measure of DNA synthesis, alkaline phosphatase specific activity, [$^3$H]-uridine incorporation as a measure of RNA synthesis, and [$^3$H]-proline incorporation into collagenase-digestible (CDPs) and non-collagenase-digestible (NCPs) proteins, and [$^{35}$S]-sulfate incorporation into proteoglycans as measures of extracellular matrix production.

Surface roughness affected cell number in a cell-specific manner. RC and MG63 cell number were decreased on rougher surfaces, while GC cell number was unaffected. [$^3$H]-thymidine incorporation by RCs and MG63s was inhibited on all surfaces compared to tissue culture plastic controls, while that by GCs was increased on rough surfaces. The level of alkaline phosphatase specific activity, a marker of cell differentiation, was also surface dependent; cultures grown on rough surfaces contained decreased amounts of enzyme specific activity. [$^3$H]-uridine incorporation by MG63s was decreased after culture on smooth surfaces. In contrast, RNA synthesis by RCs, MG63s, and GCs was increased on the rougher surfaces. There was a reduction in CDP production by RCs and MG63s on smooth surfaces, while there was no change in NCP production. GCs, in contrast, showed a reduction in both CDP and NCP production on

rough surfaces. The results for [$^{35}$S]-sulfate incorporation by all three cell types were similar, with all surfaces showing inhibition compared to controls.

The re-use of the disks after a second autoclaving had no effect on cell response The surface-dependent changes that were originally observed were obtained upon re-use of the disks.

The results of this study clearly show that surface roughness affects cell response. Surface texture and microgeometry can dramatically alter cellular response. The type of cell, and that cell's stage of maturation, are also factors in the response to a particular surface. Although further *in vivo* research is needed, these findings may aid in determining the nature of the optimal implant surface, and raises the possibility that previously used implants may be re-used.

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# I. INTRODUCTION AND LITERATURE REVIEW

## 1. Implant Requirements

Although the field of Orthopaedics has been actively researching materials suitable for implantation for many years, it is only relatively recently that research has focused into implant materials for oral reconstruction. Branemark's discovery of titanium metal incorporation into bone, plus his research into implant techniques has laid the groundwork in organizing the search for the ideal implant material. Many materials have been studied in an effort to find one that is most compatible, and one that could generate the most and quickest bone apposition. Characteristics of an ideal implant include: no inflammatory or foreign body elicited in the host, no local or systemic toxicity, integration without fibrous encapsulation, corrosion resistance, does not leach ions or particles over time, no negative influence on normal bone mineralization or formation, an ability to produce osseoinduction or osseoconduction, prevention of bacterial adhesion, and low price and easy processability (Albrektsson *et al*, 1986; Tengvall and Lundstrom, 1992). These qualities deal primarily with compatibility of the implant with the host. However, implants are required to be more than just biocompatible. They must be functionally load bearing for an extended period of time as well (Branemark, 1985). Ideally, the implant would replace the form and function of the lost tissue and interface with the host as if it were a part of the host (Gross *et al*, 1981). Hydroxyapatite and tricalcium phosphate are two materials found to have high biocompatibility and the added benefit of bioactivity in producing bone bonding (Cooley *et al*, 1992). Problems with these materials are its inherent brittleness, low impact resistance and low tensile strength, which resulted in poor *in vivo* performance (DeLange *et al*, 1989). To circumvent this weakness, different methods were developed to coat metals with hydroxyapatite in the hopes of increasing the strength while utilizing its bioactivity (Lacefield, 1988). However, other studies show a possible problem with the adherence to metal and resorption of the hydroxyapatite by the host (Matsui *et al*, 1994). Thus, long term

functioning is questionable (Lewandowski and Johnson, 1989; Jansen *et al*, 1991; Niki *et al*, 1991; Buser *et al*, 1991).

2. Use of Metals for Implants

The strength requirement for long term implant function could be fulfilled by the use of metals for implant fabrication. Metals tested included gold, stainless steel, tantalum, niobium, cobalt chromium, zirconium, titanium and titanium alloys. In comparison studies, titanium was found to be more biocompatible than tantalum or niobium (Johannson *et al*, 1990). Gold was found not to osseointegrate as well as titanium (Albrektsson and Jacobssen, 1987), and neither did stainless steel (Albrektsson and Hansson, 1986). Although bone seemed to grow to contact stainless steel at the light microscopic level, there was actually an unpredictable variation in interface ultrastructure which presented as a 500-1000nm distance between bone and the metal surface when assessed with electron microscopy (Linder *et al*, 1989) In other studies, titanium displayed higher cell proliferation rates, better bone contact and increased removal torque compared to cobalt chromium (Goldring *et al*, 1990, Puleo *et al*, 1991, Albrektsson and Johanssen, 1991). Commercially pure titanium has been shown to perform better than titanium alloy during assessment of removal torque and average bone to metal contact, as well as having a smaller interfacial zone between the bone and implant (Johansson *et al*, 1989). The comparison study of Albrektsson, *et al* (1985), on titanium and zirconium revealed good biocompatibility with both metals, with titanium considered slightly better. They noted that the interfacial zone of proteoglycans separating bone from titanium was 20-40nm while the zirconium ranged from 30-50nm. This is in contrast to a study by Listgarten *et al* (1992) where no interfacial zone existed, and bone was in direct contact with the titanium metal. Gottlander and Albrektsson (1992) have also shown that there were no significant differences in the amount of interfacial bone produced when comparing hydroxyapatite-coated and uncoated titanium implants.

3. Titanium as Implant Material

Currently, titanium is the material of choice for uncoated implants because of its biological acceptance in bone (Beder *et al*, 1956, 1957; McQuillan and McQuillan 1956; Williams 1981; Emneus *et al*, 1960), high corrosion resistance, weight compared to steel, and the fact that it can be easily prepared to any required form without inducing overt adverse hypersensitivity (Holgers *et al*, 1992), allergic, or immunologic reactions (Rae, 1981; Meachim and Pedley, 1981). Due to its high surface reactivity, titanium implants are covered by a surface oxide that is approximately 2-5 nm thick (Kasemo and Lausmaa, 1986; 1988). This is important since this implies that the titanium is never in direct contact with the host tissue. The contact is between the host tissue and the titanium oxide layer. Therefore, the biocompatibility of the implant is due not to the metal itself, but the oxide layer which forms upon it. Further studies by Hanawa (1991) have shown that the oxide layer is only one aspect when considering the biocompatibility of titanium. His studies found that titanium forms calcium phosphate layers on its passive oxide layer in neutral electrolyte solution. This calcium phosphate layer is similar to apatite, and this is responsible for its biocompatibility while the passive oxide film is responsible for the metal's corrosion resistance. In *in vivo* studies by Schwartz *et al* (1991) and Kohavi *et al* (1992) on rat tibia, it was shown that titanium enhanced the primary mineralization of bone during healing.

4. Factors Which May Affect Osseointegration

Recent studies by Steflik *et al* (1994) used light microscopy, scanning electron microscopy and transmission electron microscopy to evaluate the histomorphometry of the implant-bone interface of various titanium and ceramic dental implants placed in dog mandibles. Even after one year of loading, the clinically integrated implants presented with multiple types of tissue interfaces. Regardless of implant type, interfaces included direct apposition of bone to the implant, mineralized thick or fine fibrillar matrices against the implant, or varying thicknesses of an unmineralized matrix between the mineralized matrix and the implant. Osteoblasts could be seen apposing the implant surfaces, and becoming encased in lacunae. Foci of mineralization could be

seen in the unmineralized matrix. They report that this bony support tissue is a dynamic tissue, with approximately 35-60% of this supporting tissue around implants undergoing remodelling at any one time. Trisi *et al* (1993) presented findings from light and electron microscopies of cases where implants were removed after 7-20 years of clinical function. They describe the presence of structures similar to reversal lines at the edge of the bone side of the interface. The dynamic nature of the implant supporting tissue then implies that there are factors influencing the implant status. So what might these factors include?

The chemical composition of the metal surface must be considered when one discusses growth and apposition of bone tissue to implant surfaces. Differences between surfaces on a microscopic level will cause visible differences on a macroscopic level. This is due to the events occurring at the microscopic level which are affected by the change of surface characteristic. The role of surface composition has been examined in vitro by use of titanium films (Golijanin *et al*, 1988, 1989; Nowlin *et al*, 1989, Arai *et al*, 1988, 1989), solid titanium (Michaels *et al*,1989; Davies *et al*, 1990), and alloyed powder (Merrit and Brown, 1985; Evans and Benjamin, 1987). The results of these studies have shown that metals affect cell behavior *in vitro*. Studies using culture surfaces sputter-coated with various implant materials have also shown that surface composition affects both osteoblast and chondrocyte metabolism and phenotypic expression (Cooley *et al*, 1992, Hambleton *et al*, 1994). Athough these studies highlight the fact that titanium is the material of choice when considering implant materials, other factors still need to be considered.

Recent studies have concentrated on the effect of different surface textures (Groessner-Schreiber and Tuan, 1992) and microgeometry (Inouye *et al*, 1987; Cheroudi *et al*, 1985; Meyle *et al*, 1993) on cell attachment and growth. Wennerberg *et al* (1993) evaluated design and surface characteristics of thirteen commercially available oral implant systems using confocal scanning microscopy. They noted that design, as well as surface topography, varied considerably between the different implant systems. Thomas and Cook (1985) have shown that varying surface

texture significantly affected the interface response to the implant. A number of other variables, such as implant elastic modulus and surface composition, had no effect on bone apposition to the implant. The rough surfaced implants exhibited greater shear strengths than the corresponding smooth surfaced implants. The roughened implants also exhibited direct bone apposition, whereas the smooth implants exhibited various degrees of fibrous encasement. Michaels *et al* (1989) determined that a higher percentage of osteoblast-like cells attached to rough commercially pure titanium surfaces produced by sand blasting than to smoother surfaces polished by diamond paste. Bowers *et al* (1992) evaluated commercially pure titanium of different roughnesses and either a regular or irregular morphology. They found significantly greater cell attachment on the rough surfaces with an irregular morphology. In a histomorphometric study comparing different roughnesses of titanium implants, Buser, *et al* (1991) found that a sand blasted and acid etch surface treatment produced the most bone apposition. The results of past studies suggest that the implant surface texture is the most important parameter in determining direct bone apposition fixation. Ricci, *et al* (1991), state that the observed tissue response to smooth versus rough surfaced implants may be based on the effects of surface microgeometry on connective tissue cell behavior at the interface.

Further consideration must be given to the types of cells interacting with the implant surface (Ziats *et al*, 1988). In an *in vivo* study, Buser *et al* (1990) placed titanium implants into the mandible of monkeys where apical root portions remained. Histologic evaluation revealed that a cementum layer on the implant surface with inserting collagen fibers was achieved around implants closely approximating retained roots. Implants without contact to the roots demonstrated osseointegration. In a similar study, Piatelli *et al* (1994) placed titanium implants into the mandible of a young Landrace pig so that the implants abutted disrupted tooth buds. They noted formation of an "osteocementum" surrounding the implant which was separated from the bone by perpendicularly inserted collagen fibers. Again, areas of the implant not abutting the tooth bud revealed osseointegration. Cochran *et al* (1994) used an *in vitro* study to evaluate

attachment and growth of epithelial cells and gingival and periodontal ligament fibroblasts to titanium surfaces of different roughness. In contrast to previous studies looking at bone cells, they found that both fibroblasts had more attachment to smooth surfaces than rough surfaces. However, once attached, they proliferated well on both smooth and rough titanium surfaces. In contrast, epithelial cells attached poorly to all surfaces, and only proliferated on the smooth titanium surfaces. This suggests that the types of cells involved should be considered when selecting implant surfaces.

In intramembranous bone formation, the first interaction to the implant may be via the osteoblast. However, in endochondral bone formation, the first interaction may actually occur with chondrocytes during the earliest phases of endochondral bone formation. Another factor which may possibly influence *in vitro* study findings deals with the stage of maturation of the cells used. In primary cultures from bone explants, a population of cells is extracted which may be at various stages of maturation. Therefore, any effects from the stage of cell maturation may be lost. This should also be considered when evaluating results using a cell model .

Additional external factors which may affect successful osseointegration include implant surface contaminants (Binon *et al*, 1992), and surface treatments (Hartman *et al*, 1989; Rostlund *et al*, 1990; Smith, 1991). The presence of organic material on the surface of an implant can affect tissue response. Baier and Meyer (1988) have proposed that a scrupulously cleaned implant surface, free of contaminating overlayers, is important in ensuring implant success. Sources of contamination include the actual raw materials used in fabricating the implant, machining or shaping with tools of a dissimilar material, residues from the polishing and cleaning materials, sterilization procedures, materials leaching in during storage, contaminants picked up during surgical handling, and finally, inadvertent contact with saliva or rinse fluids during implant placement. Klauber *et al* (1990) evaluated oxide thickness and surface contaminants of six dental implants by electron spectroscopy and argon ion etching. These six implant systems were examined as packaged by the manufacturer and were not exposed to air at any time. Even so, all

implants showed oxygen, carbon and sodium present as surface contaminants. Some of the implants also were positive for the presence of nitrogen, fluorine, magnesium, silicone, chloride, calcium, manganese, tin, silver, and arsenic. This suggests that contamination of implant surfaces may have, in part, been responsible for their poor performance *in vivo*.

The method of sterilization itself appears to have a variable effect on surface contamination and the effect of different sterilization methods have been examined. Steam autoclaving may produce or leave organic contaminants on the implant surface (Doundoulakis, 1987). Further, there is some controversy whether autoclaving changes the oxide layer on titanium implants or increases oxide thickness (Keller *et al*, 1990). However, others (Machnee *et al*, 1993) have evaluated oxide composition and thickness on commercially pure titanium disks after various surface treatments and found no differences in thickness or binding energy before and after treatment.

Plasma cleaning has been noted to produce a scrupulously clean surface with high surface energy (Doundoulakis, 1987), improve surface wettability and enhance osteoblast attachment (Swart *et al*, 1992), enhance fibroblast attachment (Michaels *et al*, 1991), enhance healing by reducing the healing period (Budd *et al*, 1991), and increase corrosion resistance by increasing the surface oxide passivity (Vargas *et al*, 1992).

Ultraviolet irradiation, although capable of producing sterilization, initially had problems with "shadow zones" or "screening effects" due to the anatomic structure of the material being treated. More recently, advent of a modified germicidal arc lamp has produced what is now known as "dynamic sterilization." Sterilization procedures only require 19-20 seconds of irradiation (Singh and Schaaf, 1989; Delgado and Schaaf, 1990) UV treatment also provides a scrupulously clean surface with high surface energy (Doundoulakis, 1987), and enhances healing by decreasing the healing time (Budd *et al*, 1991).

Block *et al* (1992) have even evaluated use of an Nd:YAG dental laser on plasma-sprayed and hydroxyapatite coated implant systems. Unfortunately, not only was the laser treatment

unable to sterilize the implants against seeded organisms, but it also caused melting, loss of porosity, and other surface alterations as well.

Stanford *et al* (1994) evaluated the effects of sterilization with UV irradiation, autoclaving, ethylene oxide gas, and plasma cleaning. They noted a sterilization effect on alkaline phosphatase activity and osteocalcin production in rat bone explants, but no difference in collagen expression. To determine the effect of surface contaminants from steam autoclaving, they sterilized samples in an autoclave with a standard water source and compared it to autoclaving with ultrapure water. They found no difference in osteocalcin production between the two.

The biologic significance of these findings are not well understood at present, but it is clear that more than one parameter must be examined to determine whether surface treatments can alter cellular response. Keller *et al* (1990) reported decreased attachment of fibroblasts to autoclaved surfaces but no significant effect on cell spreading. Studies on chondrocytes (Hambleton *et al*, 1994) and osteoblasts (Windeler *et al*, 1991) have shown that both chondrocytes and osteoblasts are sensitive to surface chemistry and exhibit surface-specific responses with respect to attachment, proliferation, metabolism, differentiation, and matrix synthesis.

The implant surface may also be conditioned by the cells adjacent to it. Aparicio and Olive (1992) evaluated the chemical composition, topography, and oxide thickness of 11 failed titanium implants. While the surface oxide layer and roughness were unchanged, the superficial layer of the analyzed specimens showed an increase in the amount of carbon and silicon. This may have been due to cleaning and handling or may have been derived from adherent tissue. Regardless of the source of contamination, this study raises doubt as to the advisability of reusing retrieved implants.

For practical and financial considerations, the possibility that implants could be cleaned and re-sterilized without producing any detrimental effects on their biocompatibility or ability to support osseointegration should be explored. This may have important implications for sites of

focal osteolysis around intraoral implants and may explain the observed regeneration of bone around an oral implant after removal of the granulation tissue. Very little is known about the effect of multiple sterilization procedures on implant success. Although previous studies have shown reduced cell attachment to autoclaved surfaces compared with other sterilization methods (Veziau *et al*, 1989; Keller *et al*, 1990), this decreased cell attachment may be offset later by increased proliferation or matrix production, so that clinically, the effect may not be significant.

5. Cell Attachment Processes

In order for bone induction to occur on any substratum, several processes must occur. Initially, cells required for bone formation must arrive in the area. In the *in vitro* setting this is not a problem since the cells are pipetted directly onto the substratum. The next set of events deal with the attachment of cells to the substratum, then growth, then differentiation, bone formation. Attachment to the substratum is the first step in the process of cell/surface interactions and affects subsequent cellular and tissue responses. Cells attach to the substratum through contact sites. These are classified depending on the distance of the cell from the substratum and the presence of certain proteins inside and outside the cell (Brunette, 1988; Puleo and Bizios, 1992). Focal-type adhesions are divided into focal contacts (small transient adhesions) and focal adhesions (larger, more permanent structures composed of a number of laterally opposed focal contacts). Focal-type adhesions are characterized by a 10-15 nm separation of the ventral cell surface from the substrate and are areas where large bundles of actin-containing microfilaments from the plasma membrane terminate. Close contacts are broader areas around focal contacts characterized by a 30-50 nm gap between the cell and substrate. These have been proposed to be associated with cell spreading (Izzard and Lochner, 1980). Extracellular matrix contacts are regions where the cell and substrate are separated by large distances, >100 nm, and where the cell is in contact with extracellular components.

Previous studies have shown that a preferential adsorption of a particular protein species occurs at the titanium surface (Healy and Ducheyne, 1992). This preferential adsorption, the

resultant cell coverage, and conformational changes in native protein structure may lead to different physiologic responses. Isolation and characterization of macromolecules promoting cell attachment have resulted in identifying the following as promoters of cell attachment: fibronectin, vitronectin, osteopontin, collagens, thrombospondin, fibrinogen, laminin and epinectin. Each of these proteins have specific binding sites for certain cell types (eg. fibronectin for fibroblasts, laminin for epithelial cells, etc). These proteins have been found to contain the tripeptide arginine-glycine-aspartic acid (RGD) at their cell recognition sites. The RGD sequences are recognized by receptors on the cell surface, specifically the integrins.

Much research has been done recently in the area of integrins by Werb, *et al* (1989, 1990) and Damsky and Werb (1992). Integrins are transmembrane cell surface receptors for extracellular matrix ligands. They act as adhesion receptors and are located in focal contacts and adhesions. They allow cells to interact dynamically with the extracellular matrix molecules, promoting reorganization of the cytoskeleton, adhesive strength and cell shape in response to that extracellular matrix. Several classes of these cell surface extracellular matrix receptors have been identified by Hynes (1987). Integrins recognize most major extracellular matrix glycoproteins and some can recognize more than one extracellular component. In this way, cells may modify the strength of their interactions with extracellular matrix components by changing the types of receptors expressed. These adhesion receptors transduce signals from the extracellular matrix and from other cells that regulate gene expression and cell growth, inducing cell shape changes in response to that extracellular environment and, in turn, modulating gene expression and cell function. They can also work with growth factor receptors to transmit and integrate cellular responses to specific signals from the extracellular matrix. Therefore, expression of growth factors may be regulated by integrins. An exciting discovery and underlying hypothesis of this project is that cell shape changes induced by titanium surface roughness can induce specific gene expression and cell function in osteoblast and cartilage cells.

It is possible that the extracellular matrix binding domains of the osteoblast and cartilage cells are altered by the underlying composition and structure of the substratum (Windeler *et al*, 1991); which would in turn impact on the attachment of the cells. This can be observed on a macromolecular level. Brunette (1988) in his review article mentions four principles of cell behavior observed in cell culture to explain the interactions of cells and implants. They are as follows: 1) Contact guidance: refers to the tendency of cells to be guided in their direction of locomotion by the shape of the substratum (ie. fibroblasts and collagen fibers aligning with fine grooves). 2) Rugophilia: describes the tendency of some cells to prefer rough surfaces (ie. macrophages). 3) The two-center effect: explains the orientation of soft connective tissue cells and fibers attached to porous surfaces (ie. when implants are placed on top of fibroblasts, a cellular bridge is formed with the implant as one center and the floor of the tissue culture dish as the other). 4) Haptotaxis: describes the directional cell movement that occurs as a result of adhesive gradients on the substratum (ie. insertion of an implant creates an adhesive gradient). Cells would accumulate and attach to an implant if the implant material were more adhesive for that cell type rather than other cells or the extracellular matrix. On the other hand, cells would prefer to adhere to each other or to the extracellular matrix rather than to a low surface energy material (Cooper *et al*, 1993), resulting in capsule formation. Of course, in a closed environment, even though cells might prefer connective tissue matrix, eventual contact with the implant is inevitable.

The surface texture on an implant has the potential of specifically selecting for certain populations of cells and altering their functions. Altering surface texture may alter the surface energy which in turn alters the macromolecules adsorbing to the surface. Modification of the extracellular matrix results in alteration of gene expression and cell response. By the action of the integrins, an altered extracellular matrix produces different binding patterns which result in alteration of cell shape. This alteration of cell shape in turn modifies the cell function.

6. Investigation Purpose

Many factors are involved in implant success, and very little is known regarding the effects of these variables. Therefore, the aims of this study were multi-fold in an effort to provide some clarification into the effects of some of the afore-mentioned factors. These objectives included the following:

1) Determine the surface characteristics of five different titanium surfaces.

2) Determine the effect of steam autoclaving on the titanium oxide layer of these 5 titanium surfaces

3) Determine the effect of these five titanium surface textures on the proliferation, differentiation, matrix production, and protein synthesis of cartilage and bone cells.

4) Determine if use of the different cell types has an effect on the results.

5) Determine if the stage of cell maturation affects the results.

6) Determine if re-sterilization and re-use of the titanium surfaces produces a change in the results.

# II. MATERIALS AND METHODS

## 1. Synopsis of Experimental Protocol

An *in vitro* model was chosen over an *in vivo* one because of the following reasons: decreased experimentation time and expense, reliability, decreased animal pain and loss of life, and more direct observation of the cell-biomaterial interaction (Pizzoferrato *et al*, 1985; Goldring *et al*, 1990). In *in vivo* studies, several types of cells and chemical factors are involved in the host responses. It is a complex and multi-step process, making it difficult to assess cell responses to a specific characteristic of the implant (Reddi *et al*, 1987).

The goal of this study was to test the hypostheses that growth of osteoblast-like cells and chondrocytes on various roughnesses of titanium surfaces would produce differences in markers associated with cell proliferation, differentiation, matrix production, and protein synthesis. In addition, to determine if these results would be unchanged if the same titanium surfaces were cleaned, re-autoclaved and the same experiments run again on them.

The cartilage cells were derived from known stages of endochondral development, the resting zone (which does not mineralize in vivo) and the growth zone (in which cells hypertrophy and begin to prepare their extracellular matrix for calcification). These cells continue to express their differential phenotype in culture through four passages, including extracellular matrix synthesis, and response to hormones and growth factors. They represent cells at two distinct phases of maturation with the resting zone (RCs) being more immature than the growth zone (GCs) cells (Boyan *et al*, 1988).

The MG-63 cell line is from a human osteosarcoma, originally derived from the femur of a 14 year old male. It was first isolated by Billiau in 1975. It is a homogeneous cell line which enables us to achieve cell-specific results. It has been suggested that earlier studies with osteoblast-like cells may have given varied results due to the heterogeneity of the experimental cells (Cochran, 1990). MG-63 cells have been shown to exhibit an osteoblast-like phenotype, to produce cAMP in response to parathyroid hormone and prostaglandin E2, and to modulate alkaline phosphatase activity and osteocalcin synthesis in response to 1,25-(OH)2D3. This cell line is desirable because it remains viable and maintains its osteoblastic phenotype while proliferating through many cell passages.

2. Cell Models

A. Chondrocyte cultures:

Using a previously described method (Boyan *et al*, 1988) rib cages were removed from 125 gram Sprague-Dawley rats by sharp dissection. The resting zone and adjacent growth zone cartilages were separated and carefully dissected, maintaining the distinct cell types. The cells were plated in Dulbecco's modified Eagle's medium (DMEM) and incubated overnight in an atmosphere of 5% CO2 in air at 37° C and 100% humidity. The DMEM was replaced by two 20-minute washes of Hank's Balanced Salt Solution (HBSS), followed by sequential incubations in 0.25% trypsin (Gibco Laboratories, Grand Island, NY) and 0.02% collagenase (Type II, Worthington Biochemical Corporation, Freehold, NJ) for three hours. After enzymatic digestion of the extracellular matrix, the cells were separated from tissue debris by filtration and collected by centrifugation at 500 x g for ten minutes. The cells were resuspended in DMEM with 10% fetal bovine serum (FBS) and 50μg/ml vitamin C and plated in T75 flasks at a density of 10,000 cells/$cm^2$ for resting zone cells and 25,000 cells/$cm^2$ for growth zone cells (Rifas, 1982). These cells were then incubated for 24 hours in an atmosphere of 5% $CO_2$ at 37°C and 100% humidity. The culture media was then replaced and replaced again at 72-hour intervals. The cells for the study were subcultured three times, and fourth passage cells were used for all experiments to

ensure retention of differential phenotype prior to plating on experimental surfaces. Rat costochondral chondrocytes have been shown to maintain their phenotype at this passage level.

B. Osteoblast culture:

MG-63 cells obtained from the American Type Culture Collection (Rockville, MD) were plated at a density of 9,300 cells/$cm^2$ and grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% $CO_2$ in air at 100% humidity. Media were changed at 24 hours and then at 72-hour intervals.

3. Titanium Disk Preparation

Titanium disks were obtained from the Institute Straumann AG, Waldenburg, Switzerland. They were prepared from 1 mm sheets of grade 2, unalloyed, commercially pure titanium (ASTM F67 "Unalloyed Titanium for Surgical Implant Application" cut into disks with a diameter of 15.0 mm. Prior to treatment to produce the different surfaces, degreasing and acid prepickling of the disks were done to provide a standard starting surface. This was accomplished by washing in acetone first, then processing through 2% ammonium fluoride, 2% hydrofluoric acid-10% nitric acid solution at 55°C for 30 seconds, followed by pickling in 2% hydrofluoric acid-10% nitric acid solution at room temperature for 30 seconds. The disks were then processed to provide the different surfaces after pretreatment and consisted of the following:

1) PT: After pretreatment, rinsing was done in deionized water. Disks were neutralized in a 5% sodium bicarbonate solution, then given three 5-minute rinses in deionized water in an ultrasonic bath.

2) EP: Electropolished in an acid bath by Fluhmann AG, Wallisellen, Switzerland, using a proprietary process. This was followed by rinsing in deionized water, neutralization in 5% sodium bicarbonate solution and three five minute rinses in deionized water in an ultrasonic bath.

3) FA: Fine grit blasted with 0.12-0.25um corundum grit at 3 bar until the surface reached a uniform gray tone. Then acid etching in hydrochloric acid/ sulfuric acid (proprietary process of Institut Straumann AG, Waldenburg, Switzerland), followed by rinsing in deionized water,

neutralization in 5% sodium bicarbonate solution, and three five minute rinses in deionized water in an ultrasonic bath.

4) CA: Coarse grit blasted with 0.25-0.50um corundum grit at 3 bar until the surface reached a uniform gray tone, followed by acid etching in hydrochloric acid/ sulfuric acid (proprietary process of Institut Straumann AG, Waldenburg, Switzerland), then rinsing in deionized water prior to neutralization in 5% sodium bicarbonate solution, and two five minute rinses in an ultrasonic bath.

5) TPS: Titanium plasma sprayed (proprietary process of the Institut Straumann AG, Waldenburg, Switzerland), followed by three five minute rinses in deionized water in an ultrasonic bath.

The disks were wrapped in gauze to prevent surface damage and were sterilized by autoclaving one time in an AMSCO autoclave set at 270°F for 20 minutes at 31 $lbs/in^2$. Disks remained in the sterilization package until use. Disks were handled with titanium forceps only.

4. Surface Analysis

Surface characterization performed via collaboration with the Sony Corporation and consisted of scanning light microscopy, electron microscopy, laser confocal scanning microscopy, energy dispersion X-ray analysis, and auger electron spectroscopy. Representative disks from each group were subjected to surface analysis before and after autoclaving.

A. Light microscopy

Standard bright field microscopy was performed on each specimen with an Olympus microscope equipped with an image analysis system. Images were used for comparison with data obtained from the other surface analyses.

B. Scanning electron microscopy (SEM)

Surface characteristics of all specimens were examined at 500 to 50,000X with a JEOL 6400 FEC cold field emission scanning electron microscope (nonthermally assisted tip) with secondary and backscattered electron capability.

C. Laser confocal scanning microscopy

Confocal imaging was performed with a laser confocal scanning microscope (LSM) (Carl Zeiss, Inc., Thornwood, NY). The LSM was first calibrated for maximal confocal resolution using the 5x objective (pinhole setting of 20) and then moved to the 50x objective (pinhole setting of 0.5) for image analysis. Five areas (256μm x 256μm) were scanned on each specimen. Each specimen required a unique depth scan, due to differences in topography. The scans were visualized at a distance setting of 1 and a z:x aspect ratio = 1 (except for CA treated surfaces, where 0.982 was as close to 1 possible). Topographical analysis was then performed by five separate z-section scans.

Z-range (Rmax) is the total change in height from the lowest to the highest points for a particular z-section. The upper profile volume (Vu) is the total volume of the profile-bearing area. This volume, when added to the lower profile volume (V1), which is the excluded volume, is equal to the total scanned volume. Vu, therefore, represents the profile-bearing volume as viewed from a flat plane at the bottom of the scan. Information regarding the roughness parameters reported are the average profile height, which is the mean value (mean height) = Zm and the maximum surface roughness = Rmax.

D. Energy dispersion x-ray analysis

Energy dispersion x-ray analysis (EDX) was used to provide a bulk surface analysis to a depth of 1μm. An Amray 1645 SEM (Bedford, MA) utilizing a $LaB_6$ source was attached to a Tracor/Noran 5500 energy dispersion x-ray analyzer equipped with VISTA software (Noran Instruments, Inc., Middleton, WI) for EDX analyses. All analyses were done under the following conditions: 5,000x, 100AU at 0.010KeV for 200 seconds. Spectrographically pure (99.99%) titanium standards were used for the determination of relative values and background counts.

E. Auger electron spectroscopy

Auger electron spectroscopy (AES) was performed using a Perkin-Elmer Model 595 scanning Auger microprobe (Perkin-Elmer, Physical Electronics Division, Eden Prairie, MN). To

define the near-subsurface chemical profiles, atomic monolayers were argon-ion milled away between Auger scans at a controlled, quantified rate. The latter was established using special standards fabricated by sputter-coating pure titanium onto glass substrates; these were then fractured and inspected by SEM in order to accurately define the titanium film thickness, which was found to be 1000Å. An approximate overall ion-milling rate was defined in terms of the time (five minutes) required to remove this titanium coating (disappearance of the titanium Auger signal). It was explicitly assumed that subsequent milling of various oxidized titanium surfaces would likewise occur at the same rate of 200Å per minute.

Experiments involving treated substrates were performed by obtaining Auger spectra at regular ion-milling intervals. To quantify chemical compositions at these locations (depth beneath the surface), the heights of detected elemental peaks were measured individually, and the ratios of these to their combined height defined as the relative atomic percentage of each elemental constituent. The latter were plotted versus distance beneath the surface, which served two purposes. First, the depth of the oxide layer could be defined as the point beyond which the titanium to oxide (Ti:O) ratio exceeded two ($TiO_2$ being the highest, but not the only, stable oxidized state of titanium). Second, the near-surface elemental distributions can be extrapolated back to the surface itself, whose own Auger spectra is always contaminated by ambient environmental carbon monoxide.

5. Plating of Cells

Confluent, third passage resting zone and growth zone cartilage cells were plated to begin their fourth passage, and MG63 cells were plated on 24-well (16 mm diameter) culture plates containing the different titanium disks. Each disk type was placed into 6 separate wells (n = 6) with the last two rows on the second culture plate serving as controls. Control wells did not contain any titanium disks. The cell densities for the plates were 10,000 cells/$cm^2$ for RCs, 25,000 cells/$cm^2$ for GCs, and 9,300 cells/$cm^2$ for MG63 cells.

6. Cell Proliferation

A. Cell number

Cell number was determined at 24 and 48 hours after visual confluence of the cells in the plastic control well. At the 24 hour mark, residual media was removed by aspiration. Cells were released from the extracellular matrix by incubation in 500ul of 0.25% trypsin ethylenediamine tetra-acetic acid (EDTA) for 10 minutes at 37°C. The solution wass pipetted from the well into a 15ml test tube. The well was washed with 500ul of Dulbecco's Modified Eagle's Medium (DMEM) and the wash material pipetted into the same test tube. 500ul of full media (DMEM + 10%FBS) was added to the tube to stop the reaction. An additional 500ul of 0.25% trypsin EDTA was placed into the wells for 15 minutes at 37°C. This solution was pipetted into a separate 15ml test tube to determine if a single trypsinization treatment was sufficient to remove all the cells from the disks. Another 500ul of DMEM wash of the wells was done and pipetted into the second set of 15ml test tubes. Again, 500ul of full media was added to the tube to stop the trypsinization reaction.

The tubes were spun at 500g for 15 minutes, the supernatant poured off, and the pellet resuspended in 1ml of phosphate buffered saline (PBS). 200ul of sample was added to a coulter counter vial containing 9.8ml of 0.9% NaCl for cell count on the coulter counter.

The tubes were spun again at 500g for 15 minutes, the supernatant removed, and the cell pellet resuspended in 500ul of 0.05% triton X-100 with vigorous vortexing. The cell membranes were disrupted through a series of three freeze-thaw cycles and stored at -20°C. These samples were then assayed for alkaline phosphatase specific activity as described below, and the protein content of each cell layer harvested was determined using the Lowry assay.

This same procedure was used for determining the cell number 48 hours after the cultured cells had grown to visual confluence in the plastic control wells.

B. Mitogenesis

DNA production was determined by the incorporation of acid-insoluble [$^3$H]-thymidine (Schwartz, 1989). Cells were grown on the different titanium surfaces in full media to confluence as determined by the cells in the plastic control wells. At confluence, the cells were synchronized and made quiescent by incubating in depleted media consisting of DMEM with 1% FBS and 1% vitamin C for 48 hours.

1) To determine [$^3$H]-thymidine incorporation after 24 hours, the media was changed after the quiescence period with 500µl per well of depleted media. After 20 hours, 50 µl of [$^3$H]-thymidine (4µCi/ml) was added to the wells for 4 hours.

2) To determine [$^3$H]-thymidine incorporation after 48 hours, the media was changed after quiescence with 500µl of depleted media, and after 44 hours, 50µl of [$^3$H]-thymidine (4µ Ci/ml) was added to the wells for 4 hours.

At the end of incubation, cells were rinsed twice with 500µl of PBS. The cells were fixed to the surfaces by rinsing with 500µl of cold 5% Trichloroacetic Acid solution (TCA 5gm/100ml $H_2O$) 3 times. The third time for 30 minutes at 4°C. The TCA was removed from the wells and the wells were air dried. Fixed material was dissolved in 250µl of 1% sodium dedecyl sulfate (SDS) 1gm/100ml $H_2O$ overnight at room temperature in a humidity box to prevent evaporation of the SDS. The dissolved material was placed into a scintillation vial containing 10ml of scintillation fluid (Beckman), and radioactivity measured in a scintillation spectrophotometer.

7. Cell Differentiation

A. Alkaline phosphatase of cell layer:

Harvest occured by the method of Hale *et al* (1986) at confluence of the cells in the plastic control wells. The media was removed and the wells washed 2 times with PBS. 500µl of PBS was added per well and the wells were then scraped with a scraper or pipette tip to loosen the cells. Cells were transferred to a test tube, another 500µl of PBS was added to each well and the wells were scraped a second time. This solution was also added to the test tube. The tubes were

centrifuged for 10 minutes at 500g, the supernatant removed, and 1ml of PBS added to each tube. The tubes were spun again at 500g for 10 minutes, the supernatant removed, and 500µl of 0.05% triton X-100 was added to each tube. These samples were then used in the enzyme assays.

B. Alkaline phosphatase of cells:

The alkaline phosphatase specific activity is a known marker for mineralization and was measured as a function of release of para-nitrophenol from para-nitrophenyl phosphate at pH 10.2 (Bergmeyer, 1983).

8. Determination of Protein Synthesis

A. Protein production

Protein production was assessed by determination of RNA synthesis via incorporation of acid-insoluble [$^3$H]-Uridine. At confluence, the media was changed with 500µl per well of full media (DMEM + 10% FBS). After 5 hours, 50µl of [$^3$H]-uridine (4µCi/ml) was added for 2 hours. At the end of incubation, cells were rinsed twice with 500ul of PBS. The cells were fixed to the surfaces by adding 500µl of cold 5% TCA 3 times, the third time for 30 minutes at 4°C. The TCA was removed from the wells, and the wells air dried. Fixed material was dissolved in 250µl of 1% SDS overnight at room temperature in a humidity box to prevent evaporation of the SDS. The contents of the wells were added to scintillation vials containing 10ml of scintillation fluid (Beckman), and radioactivity was measured in a scintillation spectrophotometer.

9. Determination of Matrix Production

A. Production of collagen and non-collagen proteins

Extracellular matrix production was determined through incorporation of [$^3$H]-proline into collagenase digestible proteins (CDPs) and non-collagenase digestible proteins (NCPs) (Raisz, 1979). The percentage of collagen synthesis was calculated after multiplying the labeled proline in NCP by 5.4 to correct for the relative abundance in collagen (Beresford, 1986).

The cells were cultured to confluence in full media, then radioactively labelled for 24 hours with 5µCi/ml of [2, 3-3H]-proline (NEN Research Products, Dupont), in full media and 50

μg/ml of β-aminopropionitrile (Sigma). At harvest, media was removed from the wells and discarded. 200μl of 0.2N NaOH was added to each well and the wells scraped. The material was collected in a set of 12 x 75mm test tubes. Another 200μl of 0.2N NaOH was added to each well and the material collected after scraping in the same set of test tubes. 100μl of solution containing 100% TCA + 10% Tannic Acid was added to each tube. The tubes were centrifuged at 2000 rpm for 10 minutes, and the supernatant removed. The samples were then washed three times with 500μl of 10% TCA + 1% Tannic Acid with centrifugation as above with each wash. The samples were then washed 2 times in 1ml of cold Acetone, again with centrifugation. The pellets were then dissolved in 500μl of 0.05M NaOH.

1) Protein Content: 10μl of each sample was pipetted out for protein content determination by the method of Lowry (1955).

2) Protein Synthesis: 50μl of each sample was pipetted out and transferred into the corresponding scintillation vial containing 10ml of scintillation cocktail (Beckman), and radioactivity was then measured in the scintillation spectrophotometer.

The amount of proline incorporated into CDP and NCP was determined according to the method of Peterkofsky and Diegelmann (1971). This assay uses a mixture of proteinase free collagenases for the specific assay of radioactive collagen in the presence of other proteins. The method utilizes a reaction mixture of 60μmoles of Hepes buffer (pH 7.2), 125 μmoles of N-ethylmaleimide (NeM), 0.25μmoles of $CaCl_2$ and 25 units of Type II collagenase. The solution was prepared as follows: a) 7.83mg of NeM brought up to 100ml volume with ultrapure water. b) 13.88 mg of CaCl2 brought up to 100ml volume with ultrapure water. c) 7.15 mg of HEPES, plus 1ml NeM from above, and 100μl of CaCl2 from above, then brought up to 100ml volume with ultrapure water. d) 125 units of Collagenase was added for every 100μl of the HEPES-NeM-CaCl2 solution that was used. 100μl of this reaction solution was combined with 200μl of 0.08N HCl and 200 μl of sample in 2ml polypropylene microcentrifuge tubes. The tubes were placed in a shaking water bath at 37°C for 4 hours. The reaction was stopped by addition of 500

µl of 10% TCA + 0.5% Tannic Acid at 0°C (tubes placed on ice), and the tubes centrifuged at 400g for 5 minutes at 4°C. The first supernatant (part of the collagen protein) was transferred to a sintillation vial containing 10ml of scintillation fluid (Beckman).

The precipitate was resuspended in 500µl of 5% TCA + 0.25% Tannic Acid and allowed to sit overnight at 4°C to increase solubility. The solution was respun at 400g for 5 minutes at 4°C, and the second supernatant transferred into the same scintillation vial as the first supernatant. The two supernatants constituted the Collagen Protein.

The precipitate (the Noncollagen Protein) was resuspended with 1ml of 5% TCA +0.25% Tannic Acid, then transferred into other scintillation vials containing 10ml of scintillation fluid. All scintillation vials were read in the scintillation spectrophotometer.

B. Proteoglycan production

Determination of extracellular matrix production also utilized incorporation of [$^{35}$S]-sulfate for proteoglycan production. Proteoglycan production was determined 24 hours after confluence according to the method of O'Keefe, *et al* (1988). Four hours before harvesting, [$^{35}$S]-sulfate is added to the media to make a final concentratoin of 9µCi/ml. At harvest, the media was discarded and the wells washed with 500µl of PBS. The cell layer was collected by addition of 250µl of 0.25M NaOH and scraping of the wells. This procedure was done twice. From each tube, 50µl was taken for protein determination, according to the method of Lowry, *et al* (1955). To measure $^{35}SO_4$ incorporation, 250µl of 0.15M NaCl was added to the tubes to make a final volume of 700µl. Each sample was then dialyzed in a 12,000-14,000 molecular weight cut-off dialysis membrane against buffer containing 0.15M NaCl, 20mM $Na_2SO_4$, and 20mM $Na_2HPO_4$, at pH 7.4 and 4°C. The dialysis solution was changed every 8 hours until radioactivity of the dialysate reached background levels. The samples were then transferred to scintillation vials containing 10ml of scintillation cocktail and radioactivity was counted on the scintillation spectrophotometer.

10. Assessment of Re-sterilization and Re-use of Titanium Disks

MG63 cells were used to assess the effects of mulitple sterilization of the titanium disks. After the cells had been harvested, and the above biochemical assays had been performed, the titanium disks were washed using detergent and a test tube brush, first rinsed in water, then rinsed in distilled water, followed by ultrapure water. The disks were air dried, wrapped in gauze and autoclaved at 270°F for 20 minutes at 31 $lbs/in^2$ in the same AMSCO autoclave used previously. Disks remained in the sterilization package until use. MG63 cells were then re-plated onto the disks and the same biochemical assays were evaluated.

11. Statistical Management of Data

Surface characterizations were performed on two disks from each treatment group. For cell culture studies, data are presented from one of two replicate experiments. For any given experiment, each data point reported will represent the mean $\pm$ standard error of the mean for six samples per group. The data were analyzed by analysis of variance, and statistical significance was determined by comparing each data point to the plastic control using Bonferroni's t-test. Bonferroni's t-test was also used for comparisons between two surface treatments or between responses at 24 and 48 hours for a particular type of surface. P values $\leq 0.05$ were considered significant.

Data for the experiments on re-use of the titanium surfaces were treated differently. Titanium surface/plastic surface ratios were calculated for each experiment to compare new and used surfaces (basal levels are different between experiments, but the titanium surface/plastic surface ratio was similar in replicate experiments). The data were analyzed by analysis of variance, with significant differences between titanium and plastic surfaces determined by the Wilcoxon matched pair rank sum test. Significant differences between new and used surfaces were determined by Bonferroni's t-test ($P < 0.05$).

# III. RESULTS

1. Surface Characterization

A. Light Microscopy

The different surface treatments resulted in distinct differences in surface appearance. Disks in the EP group were very smooth and regular in appearance, while those in the PT group had a similar appearance, but the surface was rougher. FA disks had an appearance which was similar to that of the untreated side. TPS surfaces were extremely rough and very irregular in appearance, while disks in the CA group were also very rough, but less irregular. Sterilization by auclaving did not alter the appearance of the surfaces.

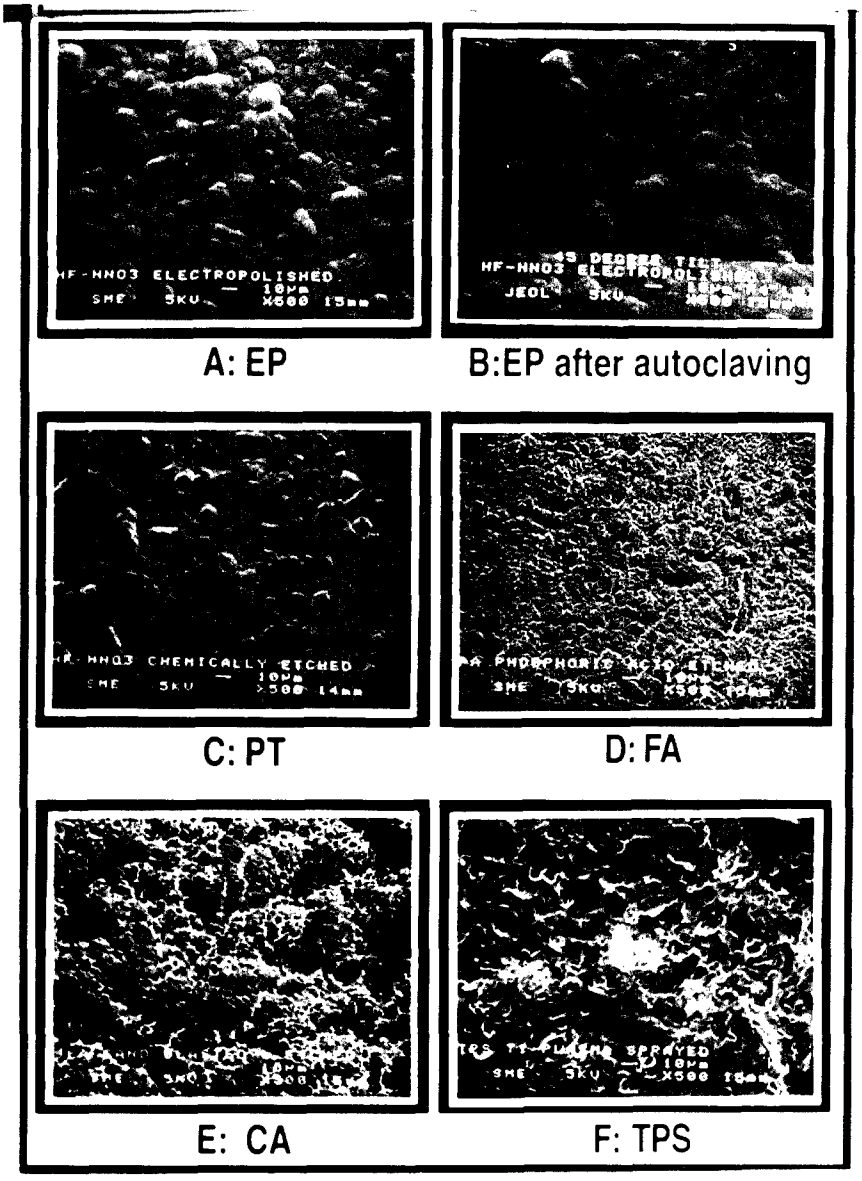
B. Scanning Electron Microscopy

SEM confirmed the smooth appearance of disks in the EP group (Figure 1A). The surface was characterized by the presence of smooth, undulating regions with occassional patches containing small crystals. Pitting of the surface was evident only at high magnification. PT surfaces were also very smooth (Figure 1B), but unlike the polished surfaces, they contained distinct grain boundaries across the surface. FA surfaces had a uniform appearance, with pits averaging 1-2μm in size; grain boundaries were seldom observed (Figure 1C). TPS-treated surfaces contained large areas of sheets and globular outcroppings, as well as deep pits, frequent fissures, and cracks (Figure 1E). Regions of irregular sharp material (<0.1μm), as well as large, smooth globules (10-20μm), could be found over the entire surface. CA surfaces contained pits and craters (Figure 1F). The pits averaged 1μm in diameter and appeared to coalesce and form large craters of 10μm in diameter. Very little evidence of grain boundaries was observed. The appearance of all surfaces was unaffected by sterilization (compare Figures 1C and 1D).

Figure 1.

SCANNING ELECTRON MICROGRAPHS OF THE FIVE TREATED SURFACES

Surfaces are shown prior to autoclaving except where noted.
A) EP
B) EP after autoclaving
C) PT
D) FA
E) CA
F) TPS


A: EP
B:EP after autoclaving
C: PT
D: FA
E: CA
F: TPS

C. Laser Confocal Scanning Microscopy

LSM largely confirmed the surface topography seen in the SEM. The height and volume data presented in Table 1 summarize the analysis of five z-scans for each specimen. Both height and volume data show that EP was the smoothest and TPS the roughest. Although the EP and PT groups appeared very similar visually, the LSM data revealed that the surface profile for PT disks was approximately twice the height and volume of EP. EP was significantly different from all other surfaces, while FA and PT, as well as PT and CA, were not significantly different. TPS disks were significantly different from all other groups in both height and volume.

D. Energy Dispersion X-ray Analysis

EDX analysis confirmed that the specimens were essentially 99.5% pure titanium. Discrete element peaks other than titanium appeared occassionally. However, they could not be regularly repeated. Comparison to known standards placed these signals in the range of background noise.

E. Auger electron Spectroscopy

For all five treatments, the dominant elements on the surface were Ti, O, and C, with trace amounts of P, Ca, K, Cl, and Na. Compared to pre-sterilized surfaces, trace elements were somewhat less abundant on the sterilized surfaces, while carbon was much higher. By exptrapolating substrate noncarbon elemental compositions to the surfaces, it was found (Table 2) that the Ti:O ratio was in the range of 0.2 and 0.4. However, it is well known that the most oxygen-rich stable oxide of titanium is $TiO_2$, which has a Ti:O ratio of 0.5. Our results reveal that the surface is rich in oxygen, with the difference in values possibly due to imprecision of quantitative estimates based on Auger signal amplitudes in multi-elemental systems. It is highly likely that the surface oxide is $TiO_2$. It's spatial extent can be estimated (Table 3) as the depth

Table 1.

LASER CONFOCAL SCANNING MICROSCOPY OF THE FIVE TREATED SURFACES

Specimens are listed from smoothest to the roughest.
Values are the mean ± S.D. of five determinations on a single disk.
*$p<0.05$ v. EP; $^{\Delta}p<0.05$ v. PT; $^{\square}p<0.05$ v. FA; $^{\#}p<0.05$ v. CA

Data were verified by analysis of a second set of disks.

| Specimens | Average Profile Height (μm) (Zm) | Range of Profile Height (μm) (Rmax) | Upper Volume Volume ($\mu m^3$) (Vu) x $10^4$ |
|---|---|---|---|
| EP | 5.02 ± 0.163 | 9.56 | 32.02 ± 1.15 |
| PT | 10.31 ± 0.423* | 19.90 | 66.64 ± 2.70* |
| FA | 9.55 ± 0.165* | 19.68 | 61.09 ± 1.19* |
| CA | 11.52 ± 0.256*□ | 21.90 | 74.64 ± 2.02*Δ□ |
| TPS | 18.28 ± 1.013*Δ□# | 39.80 | 117.92 ± 7.02*Δ□# |

Table 2.

EFFECT OF STEAM STERILIZATION ON THE TI:O RATIO OF EXPERIMENTAL SURFACES

Values are the mean ± standard deviation of five determinations on a single disk.
Data were verified by analysis of a second set of disks.

| Surface Treatment | Before Sterilization | After Sterilization |
|---|---|---|
| EP | 0.2 | 0.2 |
| PT | 0.2 | 0.3 |
| FA | 0.3 | 0.2 |
| CA | 0.3 | 0.2 |
| TPS | 0.3 | 0.4 |

below which Ti:O exceeds 0.5, or even more conservatively, in terms of the least oxygen-rich oxide, TiO, for which Ti:O = 1.

Generally, the oxygen concentration decreased with depth beneath the surface, while the relative titanium concentration increased. Based on Ti:O = 0.5 as a nominal criterion for the depth of the $TiO_2$ layer, it was found that the boundary was located at 100Å for EP or PT; 200Å for FA; 300Å for CA; and 400Å for TPS. The depth of the $TiO_2$ layer was unaffected by sterilization.

2. Cell Proliferation

A. Cell Number

Surface roughness had an effect on the cell number of all the cell types evaluated. For MG63 cells (Figure 2) twenty-four hours after confluence, the number of cells on PT, FA, and CA surfaces were similar to that on plastic controls. Significantly more cells were found on EP surfaces than on plastic, while significantly fewer cells were found on TPS surfaces. Forty-eight hours after confluence, only TPS-treated cultures contained significantly fewer cells than plastic. It was observed that a second trypsinization released additional cells from each surface (Figure 3). Twenty-four hours after confluence, significantly more cells were released from the titanium surfaces with a second trypsinization than the plastic. At forty-eight hours after confluence, a second trypsinization released a significant number of additional cells from the TPS cultures as compared to the other groups.

At twenty-four hours after confluence, GC cells on Ti surfaces was significantly decreased compared to plastic (Figure 4). In addition, there was an inverse relationship between cell number

Table 3.

EFFECT OF STEAM STERILIZATION ON TITANIUM OXIDE SURFACE DEPTH IN ANGSTROMS

Values are the mean ± standard deviation of five determinations on a single disk.
Data were verified by analysis of a second set of disks.

*N.D. = not determined

| | Depth of Surface Ti:O ≥ 1 | | Depth of Surface Ti:O ≥ 2 | |
|---|---|---|---|---|
| Surface Treatment | Before Sterilization | After Sterilization | Before Sterilization | After Sterilization |
| EP | 50 | 50 | 100 | 100 |
| PT | 50 | 50 | 100 | 100 |
| FA | 100 | 100 | 200 | 200 |
| CA | 100 | 100 | 300 | 300 |
| TPS | 400 | N.D. | 400 | N.D. |

Figure 2.
EFFECT OF TITANIUM DISK SURFACE ON MG63 CELL NUMBER AFTER THE FIRST TRYPSINIZATION.

MG63 cells were cultured on plastic or titanium disks for 24 or 48 hours after reaching confluence on plastic.
Values are the mean ± standard error of the mean of six cultures.
*$p<0.05$, titanium disk v. plastic; $^{\#}$24 v. 48 hours within a particular surface group; $^{\bullet}$TPS v, all other surfaces.

Data are from one of two replicate experiments.

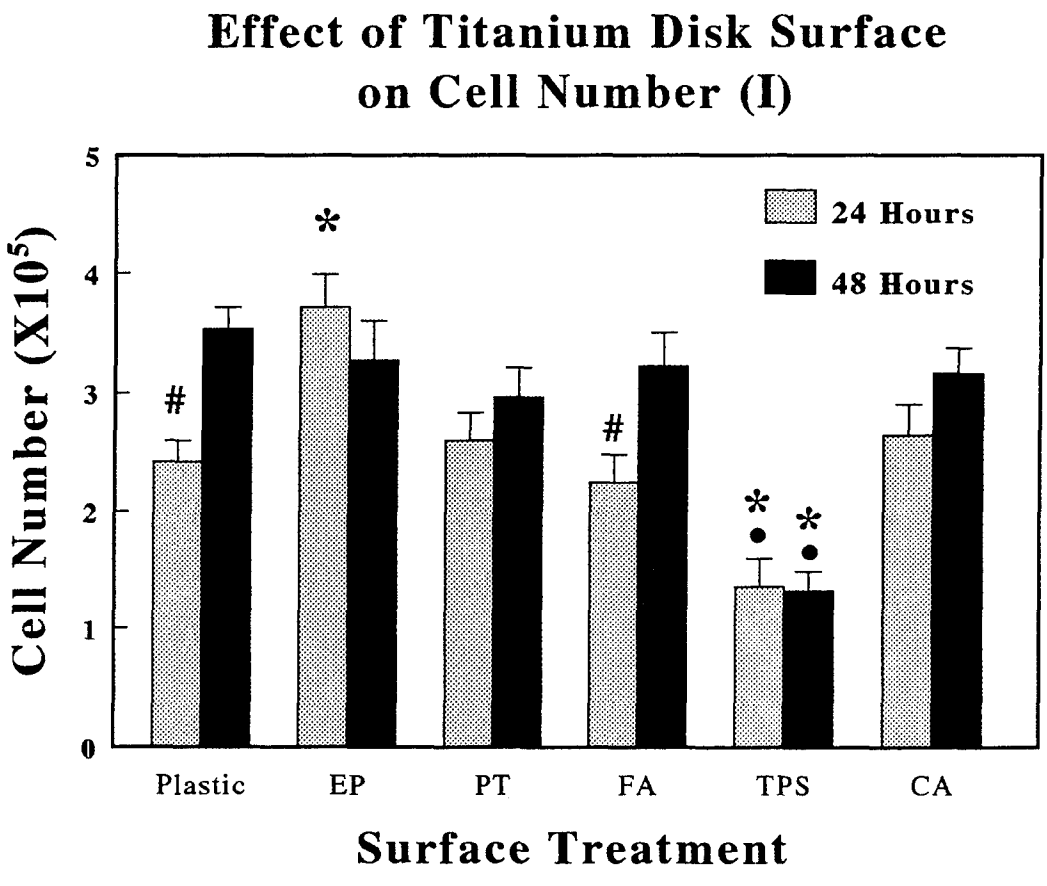

Effect of Titanium Disk Surface
on Cell Number (I)
Cell Number (X10$^5$)
0
1
2
3
4
5
24 Hours
48 Hours
#
*
#
*
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 3.

EFFECT OF TITANIUM DISK SURFACE ON MG63 CELL NUMBER AFTER THE SECOND TRYPSINIZATION.

MG63 cells were cultured on plastic or titanium disks for 24 or 48 hours after cells reached confluence on plastic.
Values are the mean ± standard error of the mean of six cultures. *$p<0.05$, titanium disks v. plastic at 24 hours; $^{\#}$24 v. 48 hours within a particular surface group; TPS v. all other surfaces at 48 hours.

Data are from the same experiment shown in Figure 2 and were verified by performing a second replicate experiment.

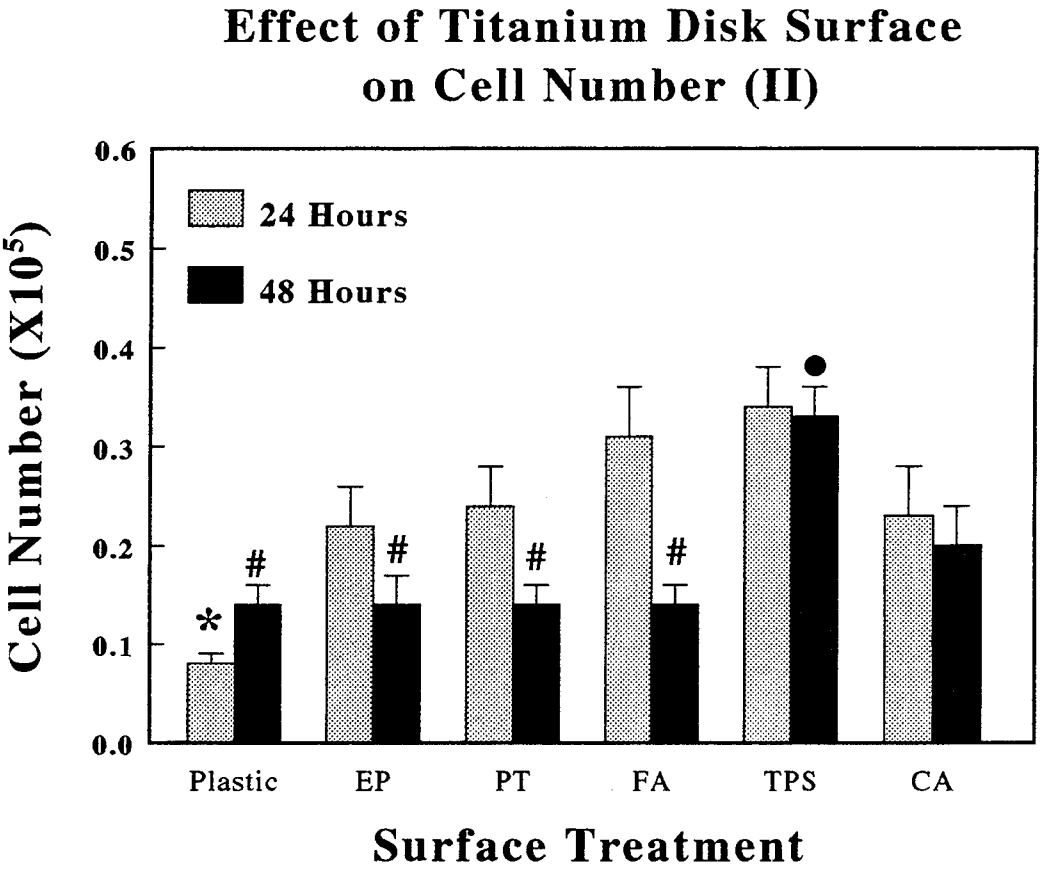
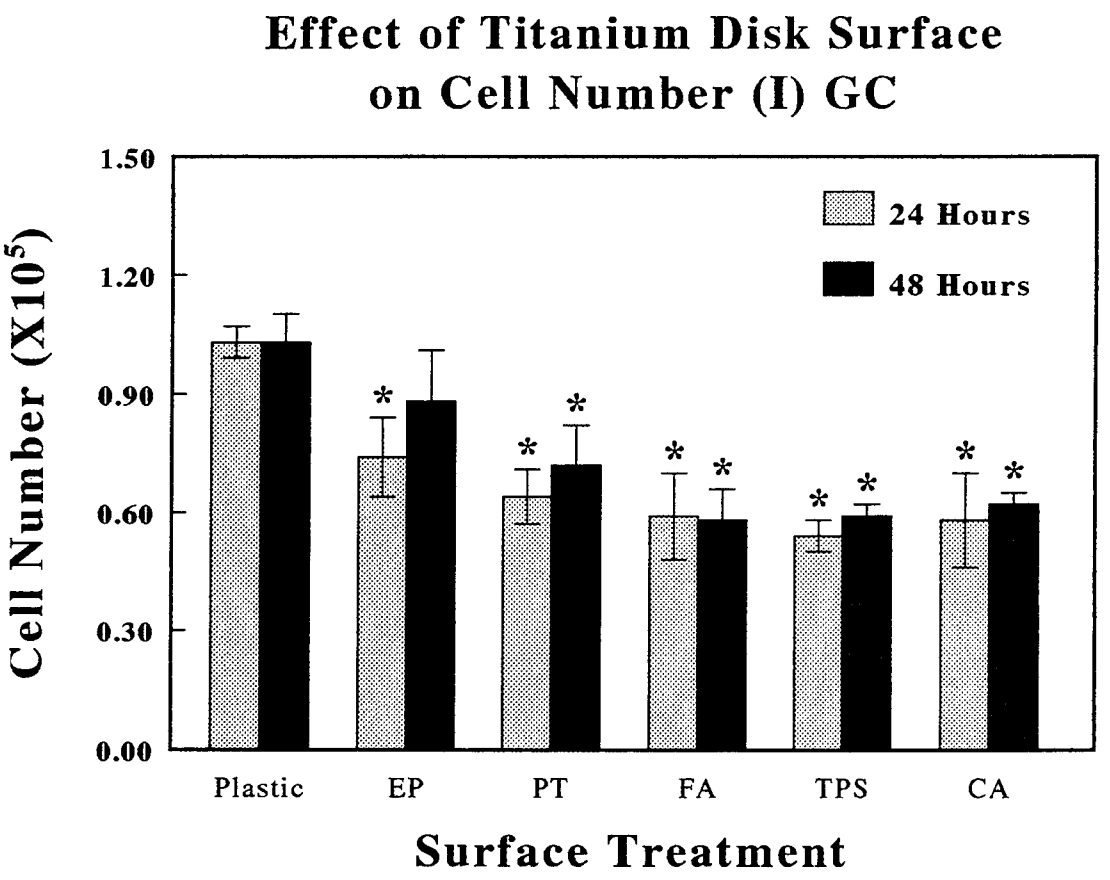

Effect of Titanium Disk Surface on Cell Number (II)
Cell Number (X10$^5$)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
24 Hours
48 Hours
*
#
#
#
#
●
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 4.

EFFECT OF TITANIUM DISK SURFACE ON GC CELL NUMBER AFTER THE FIRST TRYPSINIZATION.

GC cells were cultured on plastic or titanium disks for 24 or 48 hours after the cells had reached confluence on plastic.
Values shown are the mean ± standard error of the mean of six cultures. *$p<0.05$, plastic v. all titanium disks.

Data are from one of two replicate experiments.


Effect of Titanium Disk Surface
on Cell Number (I) GC
Cell Number (X10$^5$)
1.50
1.20
0.90
0.60
0.30
0.00
24 Hours
48 Hours
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

and surface roughness; that is, as Ti surface roughness increased, the number of cells isolated from that surface decreased. At forty-eight hours, a similar effect of surface roughness on cell number was observed, although no difference between plastic and EP surfaces was observed.

It was observed that a second trypsinization of the plastic or Ti disks released additional GC cells (Figure 5). Twenty-four hours after confluence, a second trypsinization released significantly more cells from the Ti surfaces than from the plastic. At 48 hours after confluence, however, the second trypsinization only released a significant number of cells from the rougher surfaces (FA, TPS, and CA). When RC cells were plated on the different surfaces, an effect similar to that seen with GC cells was observed after the first trypsinization (Figure 6). However, RC cell number was only two-thirds that found with the GCs. Moreover, the number of cells released by the second trypsinization was less than half that of the GC cells, and no effect of surface on cell number was observed with the second trypsinization (Figure 7).

B. [$^3$H]-Thymidine incorporation

There was a significant decrease in [$^3$H]-thymidine incorporation on all treated titanium surfaces compared to plastic (Figure 8). At twenty-four hours, [$^3$H]-thymidine incorporation by cells cultured on FA or TPS surfaces was significantly less than that seen on any of the other surfaces. By forty-eight hours, cells cultured on TPS exhibited lower values than seen on EP. In all cultures, [$^3$H]-thymidine incorporation was significantly less at forty-eight hours than at twenty-four hours.

GCs grown on different Ti surfaces showed a direct correlation between surface roughness and [$^3$H]-thymidine incorporation (Figure 9). A significant increase in [$^3$H]-thymidine incorporation by GCs was observed on FA, TPS, and CA surfaces at both 24 and 48 hours after

Figure 5.

EFFECT OF TITANIUM DISK SURFACE ON GC CELL NUMBER AFTER THE SECOND TRYPSINIZATION

GC cells were cultured on plastic or titanium disks for 24 or 48 hours after the cells had reached confluence on plastic.
Values shown are the mean ± standard error of the mean of six cultures. *$p<0.05$, plastic v. all titanium disks.

Data are from one of two replicate experiments.

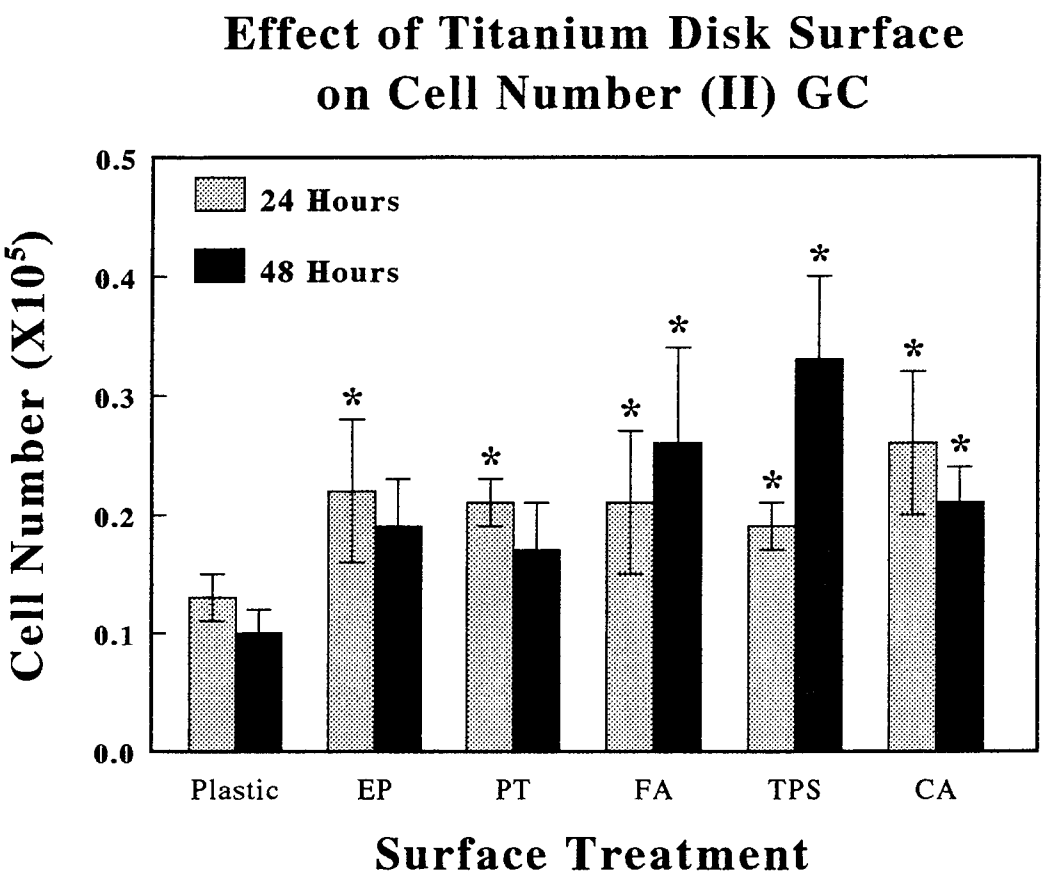

Effect of Titanium Disk Surface
on Cell Number (II) GC
Cell Number (X10$^5$)
0.5
0.4
0.3
0.2
0.1
0.0
24 Hours
48 Hours
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 6

EFFECT OF TITANIUM DISK SURFACE ON RC CELL NUMBER AFTER THE FIRST TRYPSINIZATION

RC cells were cultured on plastic or titanium disks for 24 or 48 hours after the cells had reached confluence on plastic.
Values shown are the mean ± standard error of the mean of six cultures. *$p<0.05$, plastic v. all titanium disks.

Data are from one of two replicate experiments.

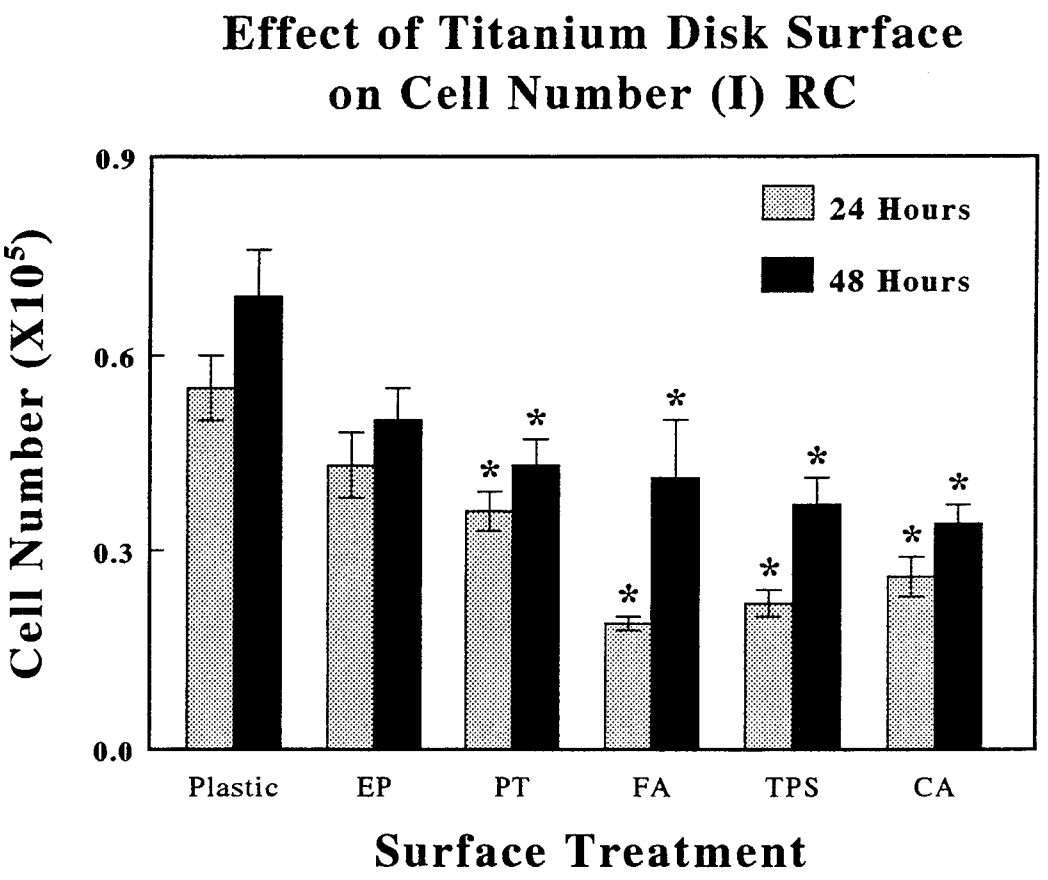

Effect of Titanium Disk Surface
on Cell Number (I) RC
Cell Number (X10$^5$)
0.9
0.6
0.3
0.0
24 Hours
48 Hours
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 7.

EFFECT OF TITANIUM DISK SURFACE ON RC CELL NUMBER AFTER THE SECOND TRYPSINIZATION

RC cells were cultured on plastic or titanium disks for 24 or 48 hours after the cells had reached confluence on plastic.
Values shown are the mean ± standard error of the mean of six cultures. *$p<0.05$, plastic v. all titanium disks.

Data are from one of two replicate experiments.

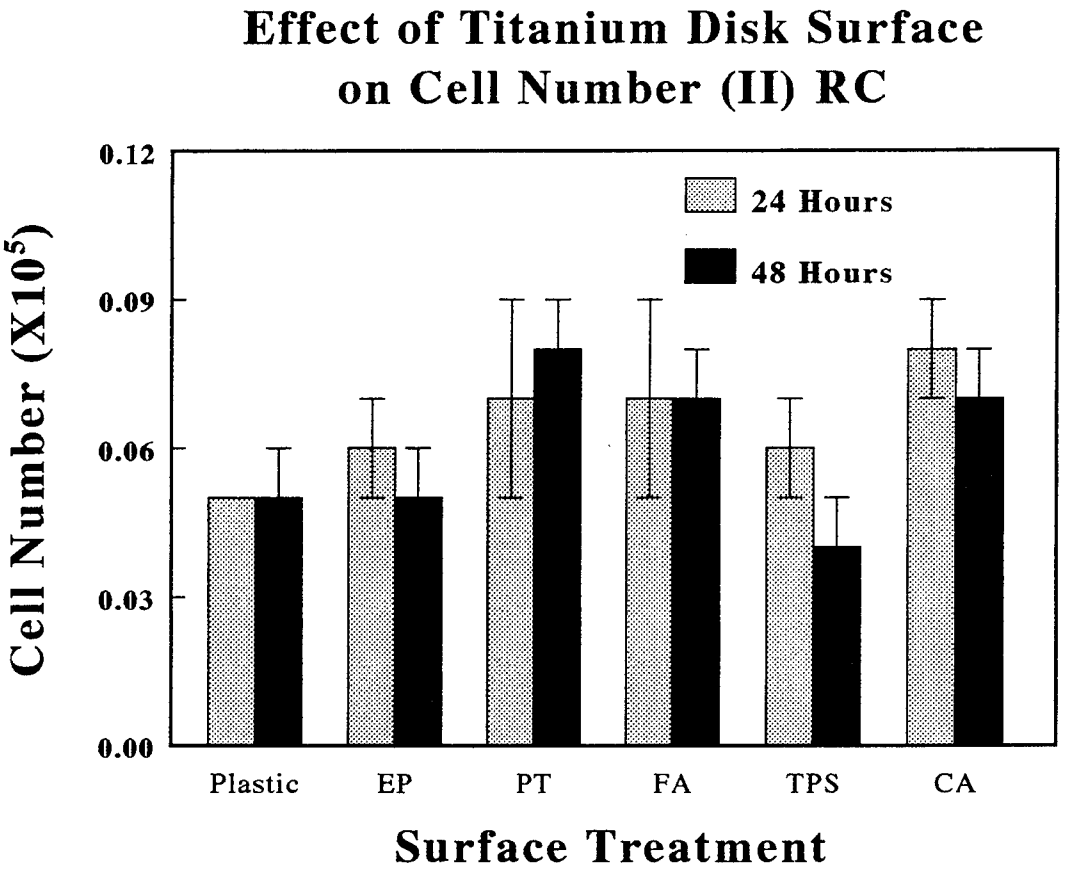

Effect of Titanium Disk Surface
on Cell Number (II) RC
0.12
0.09
0.06
0.03
0.00
Cell Number (X10$^5$)
24 Hours
48 Hours
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 8.

EFFECT OF TITANIUM DISK SURFACE ON MG63 [$^3$H]-THYMIDINE INCORPORATION

Values are the mean ± standard error of the mean of six cultures. *$p<0.05$, titanium disks v. plastic for either the 24- or 48-hour groups; $^{\#}$24 v. 48 hours within a particular surface group; $^{\bullet}$TPS or FA v. all other surfaces at 24 hours; $^{\Delta}$TPS v. EP at 48 hours.

Data are from one of two replicate experiments.

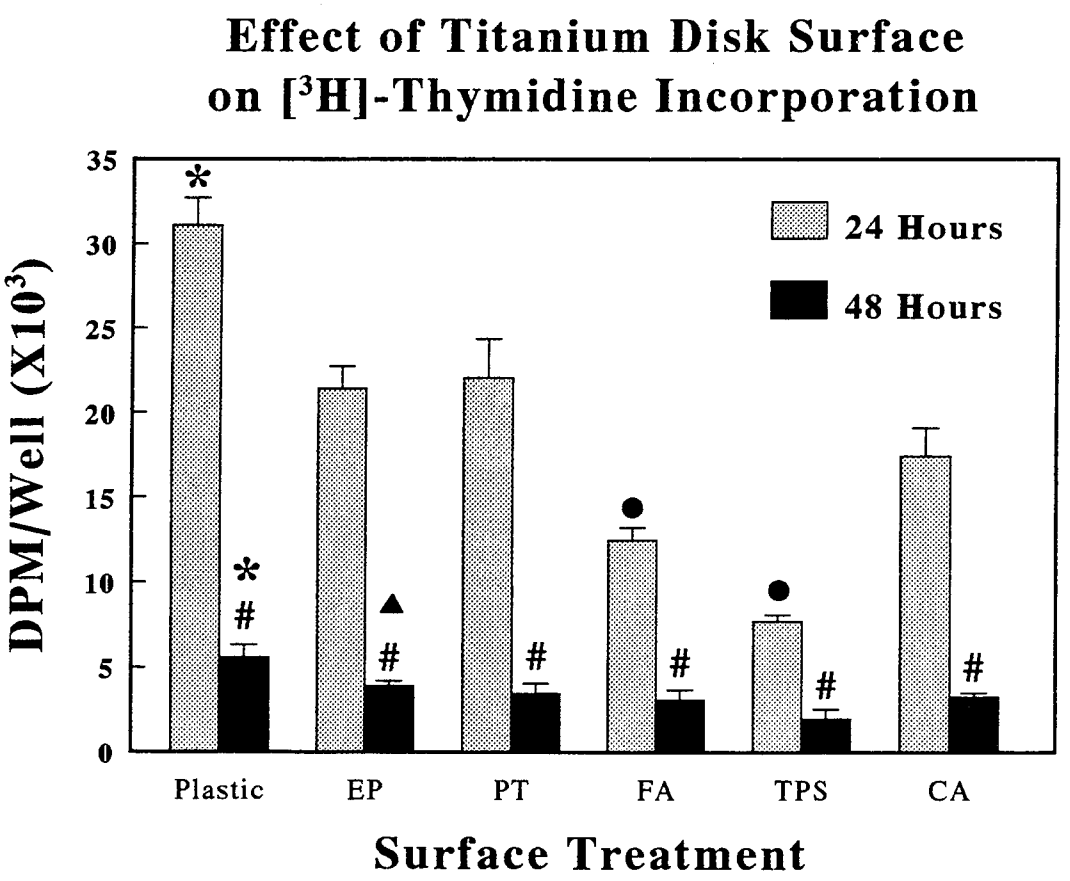
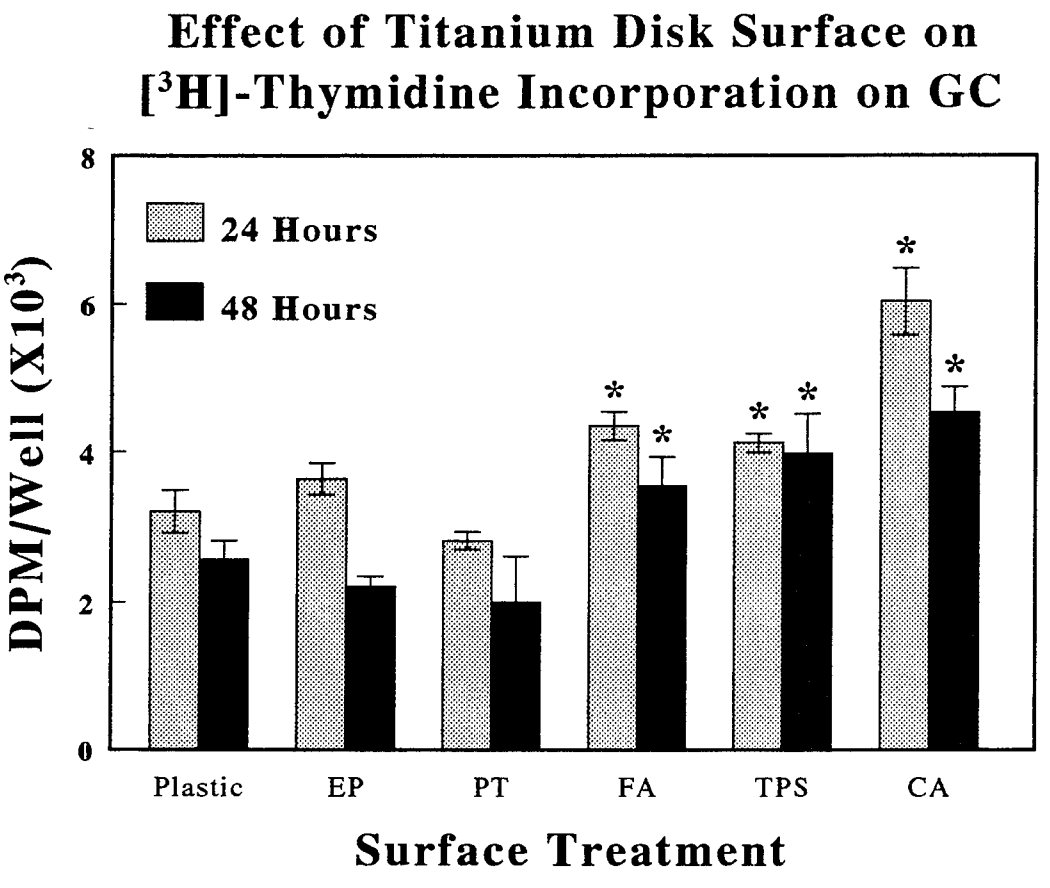

Effect of Titanium Disk Surface
on [3H]-Thymidine Incorporation
DPM/Well (X10^3)
35
30
25
20
15
10
5
0
24 Hours
48 Hours
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 9.

EFFECT OF TITANIUM DISK SURFACE ON GC [$^3$H]-THYMIDINE INCORPORATION

Values shown are the mean ± standard error of the mean of six cultures.
*Significant difference plastic v. all titanium surfaces at $p<0.05$ level.

Data are from one of two replicate experiments.


Effect of Titanium Disk Surface on
[3H]-Thymidine Incorporation on GC
24 Hours
48 Hours
DPM/Well (X10^3)
0
2
4
6
8
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

confluence. In contrast, when RCs were plated on similarly prepared Ti surfaces, an inverse relationship between surface roughness and [$^3$H]-thymidine incorporation, at both 24 and 48 hours, was observed (Figure 10).

3. Cell Differentiation

A. Alkaline phosphatase specific activity

Alkaline phosphatase specific activity varied with cell type and culture surface. In MG63 cells (Figure11), cell layers from cells cultured on FA or TPS surfaces contained less enzyme specific activity than those from cultures grown on plastic or the other treated titanium surfaces. No other differences in enzyme specific activity were found among the surfaces.

When ezyme activity of isolated cells (Figure 12) was measured after culture on FA-, TPS-, or CA-treated surfaces for twenty-four hours after confluence, an inhibition in alkaline phosphatase specific activity was observed compared to plastic. Further, enzyme activity of cells cultured on TPS and CA surfaces was significantly less than on all other titanium surfaces. At forty-eight hours after confluence, enzyme specific activity of cells cultured on TPS and CA remained lower than that observed on plastic and PT. Alkaline phosphatase specific activity of cells culture on CA was also lower than that of cells cultured on EP. Between twenty-four and forty-eight hours, activity decreased in cells cultured on plastic, as well as on EP- and FA-treated titanium.

Cell layers of GCs cultured on TPS and RCs cultured on FA, TPS, and CA contained less enzyme specific activity than those from cultures grown on plastic or the other titanium surfaces. No other differences in enzyme specific activity were found with the other surfaces (Figure 13).

Figure 10.

EFFECT OF TITANIUM DISK SURFACE ON RC [$^3$H]-THYMIDINE INCORPORATION

Values shown are the mean ± standard error of the mean of six cultures.
*Significant difference plastic v. all titanium surfaces at $p<0.05$ level.

Data are from one of two replicate experiments.

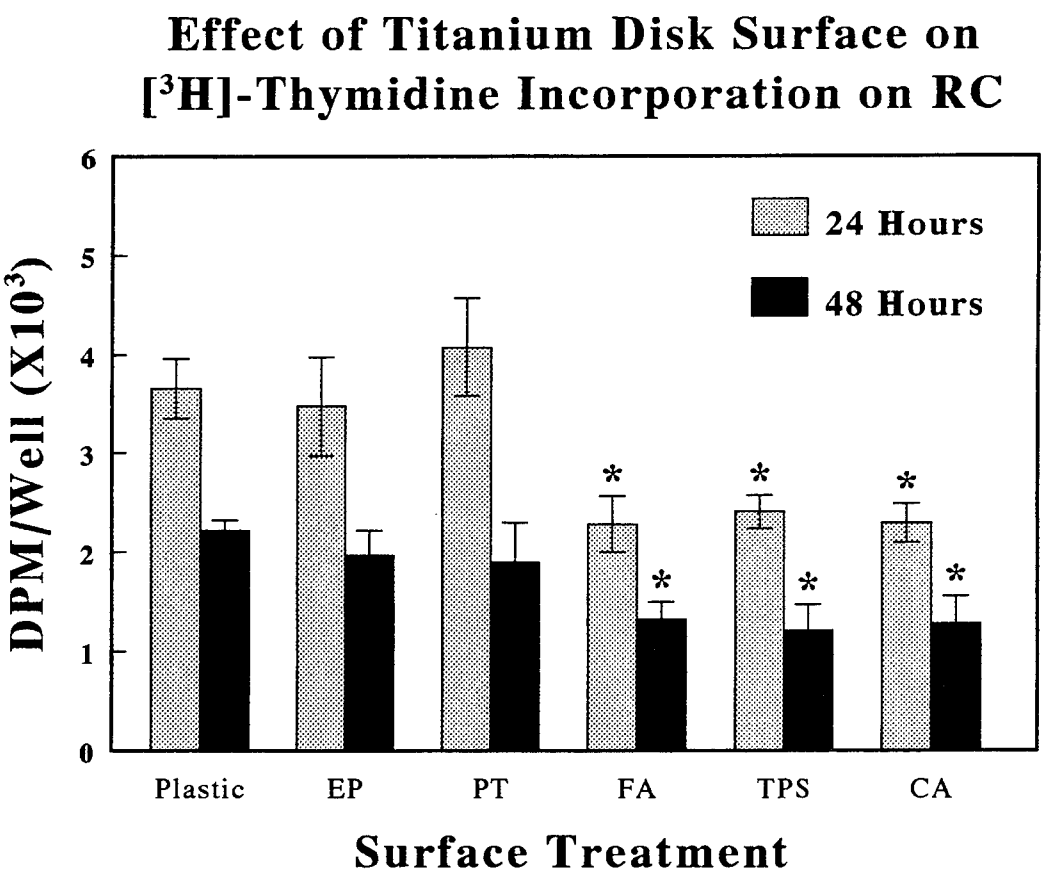

Effect of Titanium Disk Surface on
[3H]-Thymidine Incorporation on RC
DPM/Well (X10^3)
0
1
2
3
4
5
6
24 Hours
48 Hours
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 11.

EFFECT OF TITANIUM DISK SURFACE ON MG63 CELL LAYER ALKALINE PHOSPHATASE ACTIVITY

Cells were harvested by scraping the cell layer 24 hours after confluence on plastic.
Values are the mean ± standard error of the mean of six cultures.
*$p<0.05$: FA- and TPS-treated disks v. all other surfaces.

Data are from one of two replicate experiments.

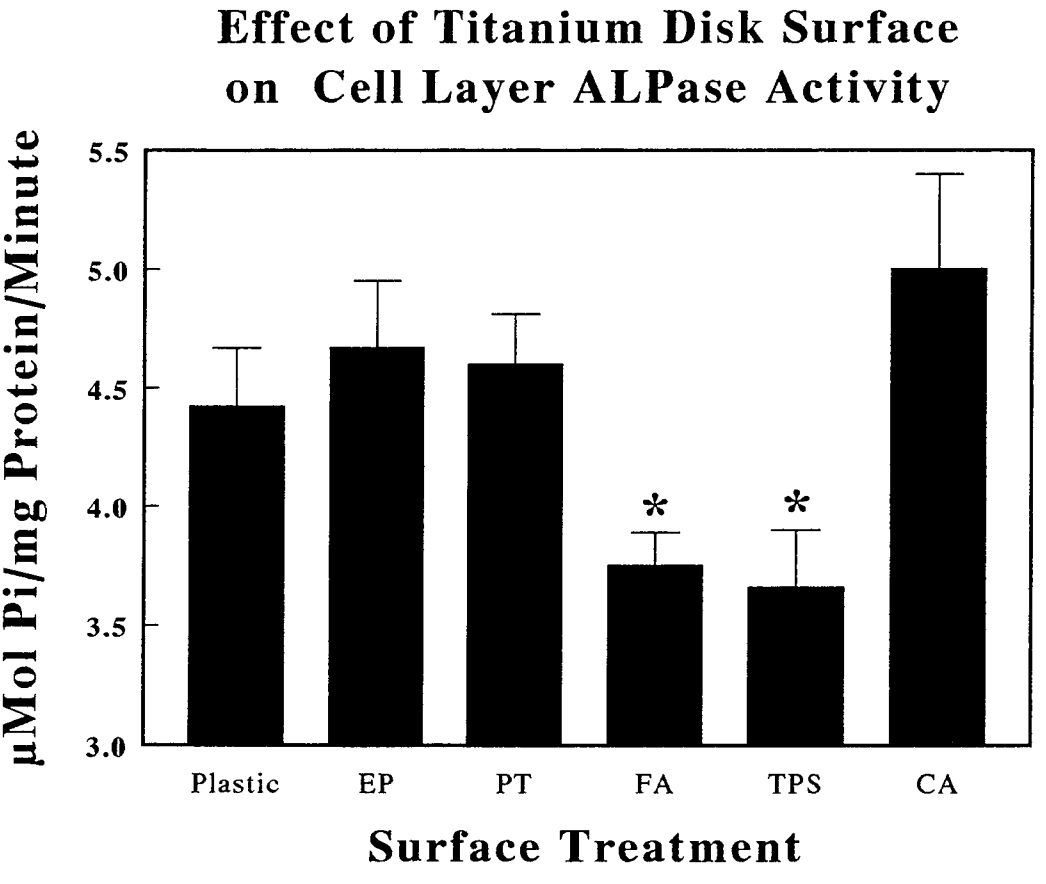

Effect of Titanium Disk Surface
on Cell Layer ALPase Activity
µMol Pi/mg Protein/Minute
5.5
5.0
4.5
4.0
3.5
3.0
*
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 12.

EFFECT OF TITANIUM DISK SURFACE ON MG63 CELL ALKALINE PHOSPHATASE ACTIVITY

Cells were harvested by trypsinization 24 or 48 hours after confluence on plastic.
Values are the mean ± standard error of the mean of six cultures.
*$p<0.05$, titanium v. plastic, $^{\#}$24 v. 48 hours within a particular surface group; $^{\bullet}$TPS and CA v. all other surfaces at 24 hours; $^{\Delta}$TPS v. PT at 48 hours; ■CA v. PT, or EP at 48 hours.

Data shown are from one of two replicate experiments.

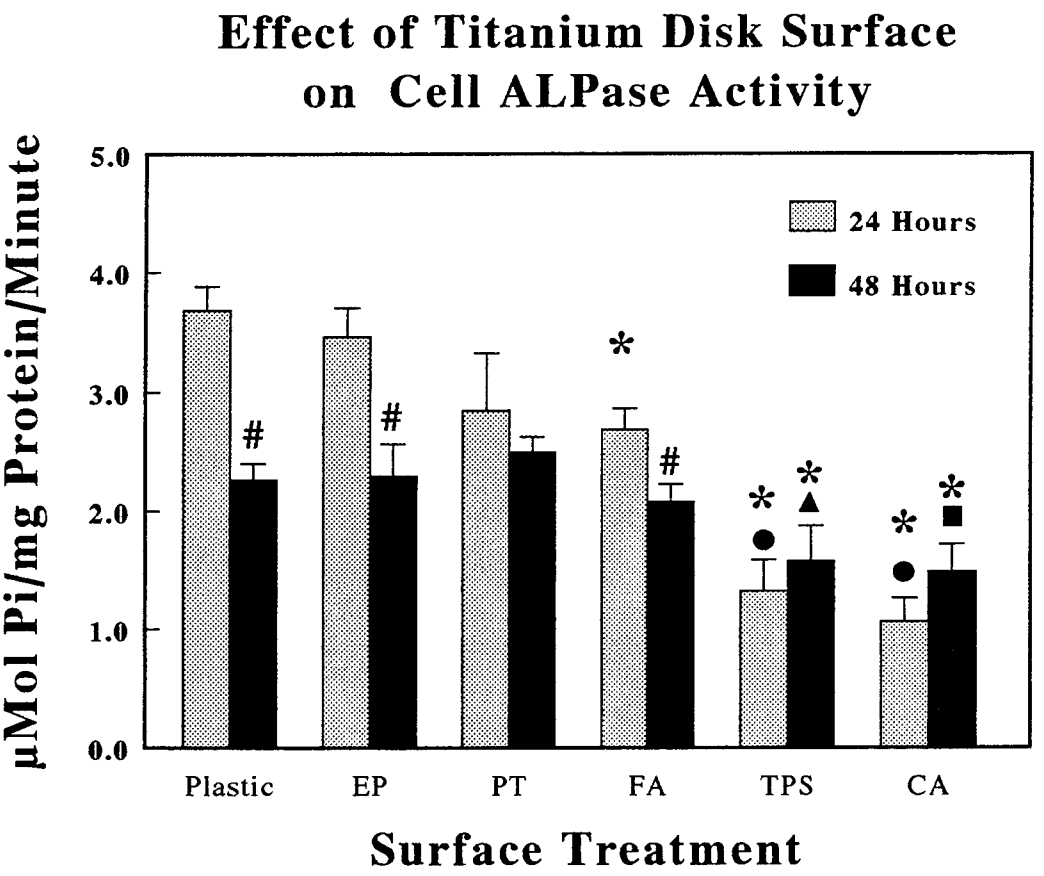
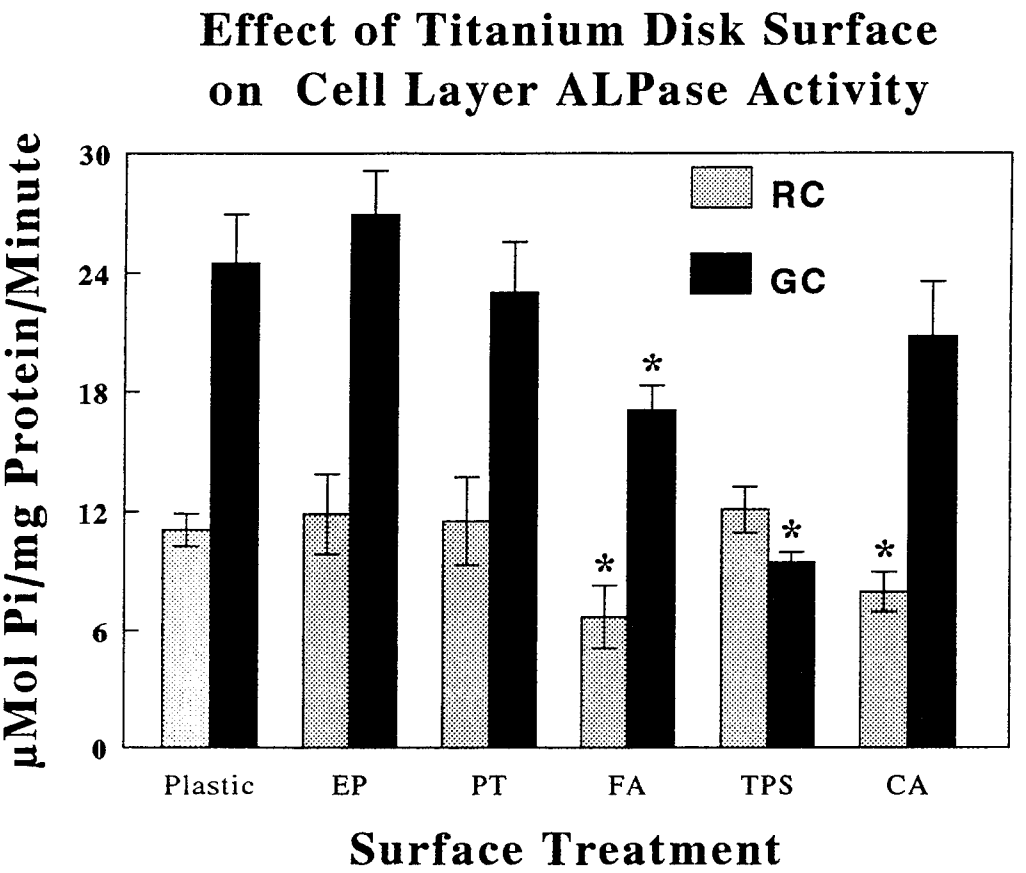

Effect of Titanium Disk Surface
on Cell ALPase Activity
µMol Pi/mg Protein/Minute
5.0
4.0
3.0
2.0
1.0
0.0
24 Hours
48 Hours
#
#
*
#
*
*
*
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 13.

EFFECT OF TITANIUM DISK SURFACE ON RC AND GC CELL LAYER ALKALINE PHOSPHATASE ACTIVITY

Cells were harvested by scraping the cell layer 24 hours after confluence on plastic.
Values shown are the mean ± standard error of the mean of six cultures.
*$p<0.05$, FA- and TPS-treated titanium disks v. all other disks.

Data shown are from one of two replicate experiments.


Effect of Titanium Disk Surface
on Cell Layer ALPase Activity
µMol Pi/mg Protein/Minute
30
24
18
12
6
0
RC
GC
*
*
*
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

When alkaline phosphatase specific activity in isolated GC cells was measured, an inhibition of enzyme activity was only found in cells that had been cultured on TPS and CA surfaces. This effect was found at both 24 and 48 hours (Figure 14). RCs showed a similar inhibition of cell alkaline phosphatase activity after culture on PT, FA, TPS, and CA surfaces. After 48 hours, the reduced activity was only found on FA, TPS, and CA surfaces (Figure 15).

4. Protein Production

[$^3$H]-Uridine incorporation results varied with the cell type examined as well as with surface characteristic. MG63 cells showed an inhibition on the smoother surfaces compared to control while the rough surfaces were either unaffected or showed an increase compared to control. RCs and GCs behaved similarly to each other in showing an increase in [$^3$H]-Uridine incorporation on rough surfaces.

[$^3$H]-Uridine incorporation by MG63 cells cultured on plastic and CA-treated titanium was comparable (Figure 16), while that of cells cultured on EP-, PT-, or FA-treated surfaces was decreased. In TPS cultures, incorporation was higher than that found on EP-, PT-, or FA-treated surfaces. It was also higher than that found on CA-treated surfaces, but this was not significant. [$^3$H]-Uridine incorporation by cells cultured on FA-treated disks was greater than that seen on EP surfaces, while [$^3$H]-Uridine incorporation on CA was greater than seen on either EP- or PT-treated surfaces.

[$^3$H]-Uridine incorporation by RCs was significantly increased by culture on FA, TPS, and CA surfaces, while that by GCs was only increased on TPS and CA surfaces (Figure 17).

Figure 14.

EFFECT OF TITANIUM DISK SURFACE ON GC CELL ALKALINE PHOSPHATASE ACTIVITY

Cells were harvested by trypsinization 24 or 48 hours after confluence on plastic.
Values shown are the mean ± standard error of the mean of six cultures.
*$p<0.05$, plastic v. all titanium surfaces.

Data shown are from one of two replicate experiments.

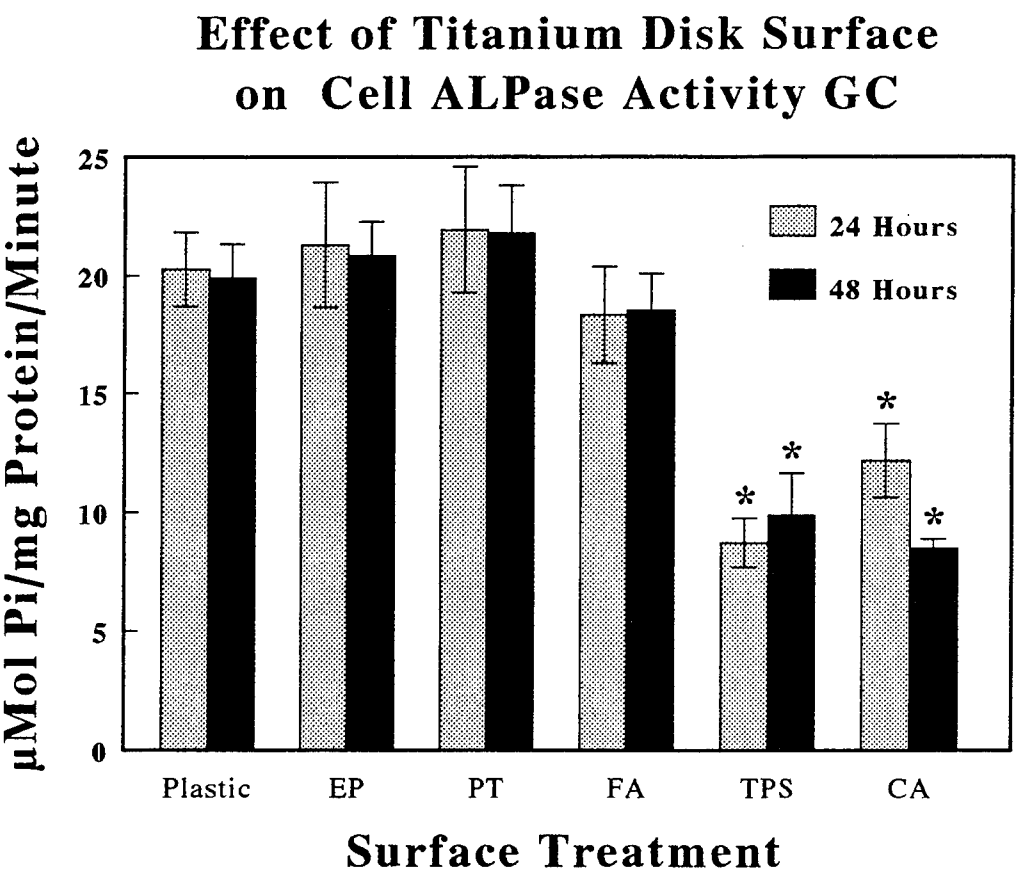

Effect of Titanium Disk Surface
on Cell ALPase Activity GC
µMol Pi/mg Protein/Minute
25
20
15
10
5
0
24 Hours
48 Hours
*
*
*
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 15.

EFFECT OF TITANIUM DISK SURFACE ON RC CELL ALKALINE PHOSPHATASE ACTIVITY

Cells were harvested by trypsinization 24 or 48 hours after confluence on plastic.
Values shown are the mean ± standard error of the mean of six cultures.
*$p<0.05$, plastic v. all titanium surfaces.

Data shown are from one of two replicate experiments.

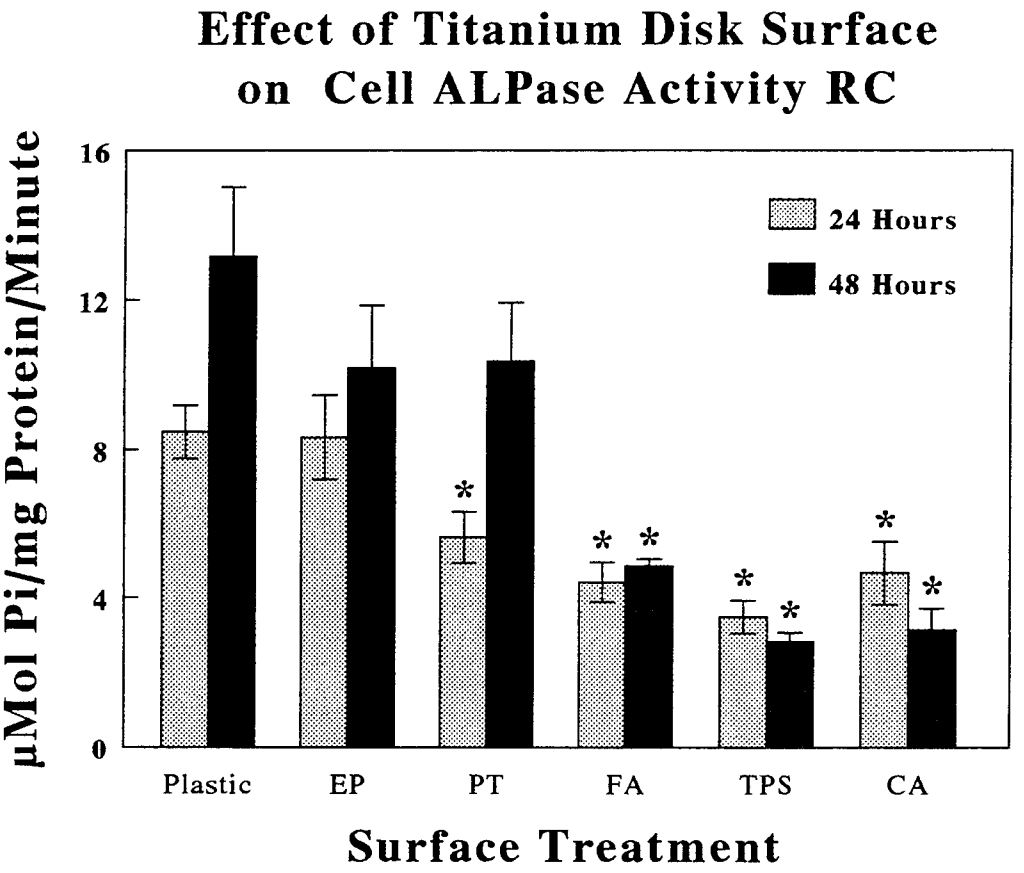

Effect of Titanium Disk Surface
on Cell ALPase Activity RC
µMol Pi/mg Protein/Minute
16
12
8
4
0
24 Hours
48 Hours
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 16.

EFFECT OF TITANIUM DISK SURFACE ON MG63 [$^3$H]-URIDINE INCORPORATION

Values are the mean ± standard error of the mean of six cultures.

*$p<0.05$, plastic v. all titanium disks except CA; $^{\#}$CA v. PT or EP; $^{\bullet}$TPS v FA, PT, or EP; $^{\Delta}$FA v. PT or EP.

Data are from one of two replicate experiments.

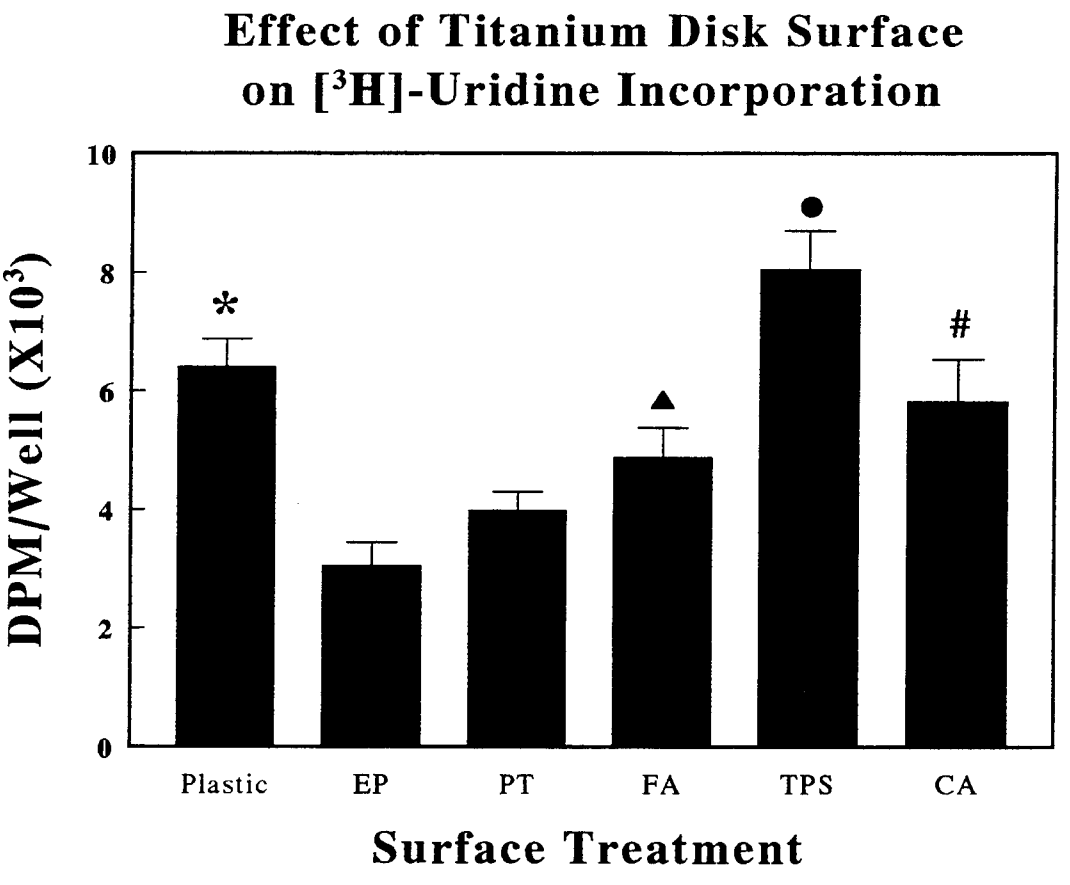
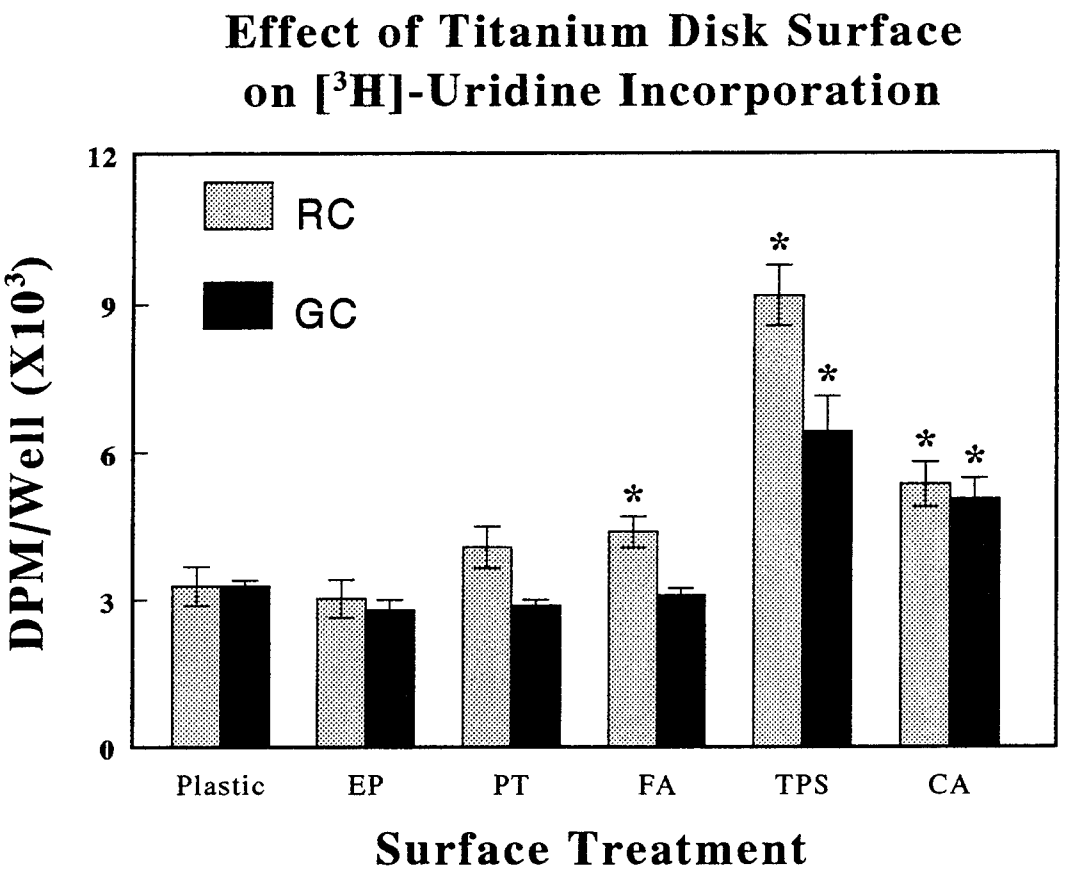

Effect of Titanium Disk Surface
on [3H]-Uridine Incorporation
DPM/Well (X10^3)
10
8
6
4
2
0
*
▲
●
#
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 17.

EFFECT OF TITANIUM DISK SURFACE ON GC AND RC [$^3$H]-URIDINE INCORPORATION

Values shown are the mean ± standard error of the mean of six cultures.
*$p<0.05$, plastic v. all titanium surfaces.

Data are from one of two replicate experiments.


Effect of Titanium Disk Surface
on [3H]-Uridine Incorporation
DPM/Well (X10^3)
12
9
6
3
0
RC
GC
*
*
*
*
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

5. Matrix Synthesis

A. Collagen and Non-Collagen Proteins

CDP and NCP production displayed a variable response by cell type to the different surface textures. MG63 cells and RCs showed similar responses in revealing a reduction in CDP production on smoother surfaces, whereas GCs showed showed a reduction on the rougher surfaces.

In MG63s, CDP production by cells cultured on EP- and PT-treated surfaces was significantly reduced when compared to all other surfaces, including plastic (Figure 18). NCP production by the cells was not affected by culturing on the different surfaces. Percent collagen production was decreased in cultures grown on EP-, PT-, and FA-treated surfaces compared to plastic (Figure 19). Collagen production on EP was slightly greater than on PT, but less than on the other treated titanium disks. Production on PT was also less than on the other treated surfaces.

GCs cultured on FA, TPS, and CA surfaces showed a significant inhibition of CDP production; inhibition of NCP production by GCs was observed after culture on TPS and CA surfaces (Figure 20). In contrast, CDP production by RCs was only inhibited after culture on EP and PT surfaces. The different surfaces had no effect on NCP production by RC cells (Figure 21). The percent collagen production by RCs was inhibited after culture on EP and PT surfaces; GCs, on the other hand, showed a significant increase in percent collagen production after culture on FA, TPS, and CA surfaces (Figure 22).

Figure 18.

EFFECT OF TITANIUM DISK SURFACE ON MG63 [$^3$H]-PROLINE INCORPORATION FOR CDP AND NCP PRODUCTION

Values shown are the mean ± standard error of the mean of six cultures.
*$p<0.05$, EP or PT v. all other surfaces.

Data are from one of two replicate experiments.

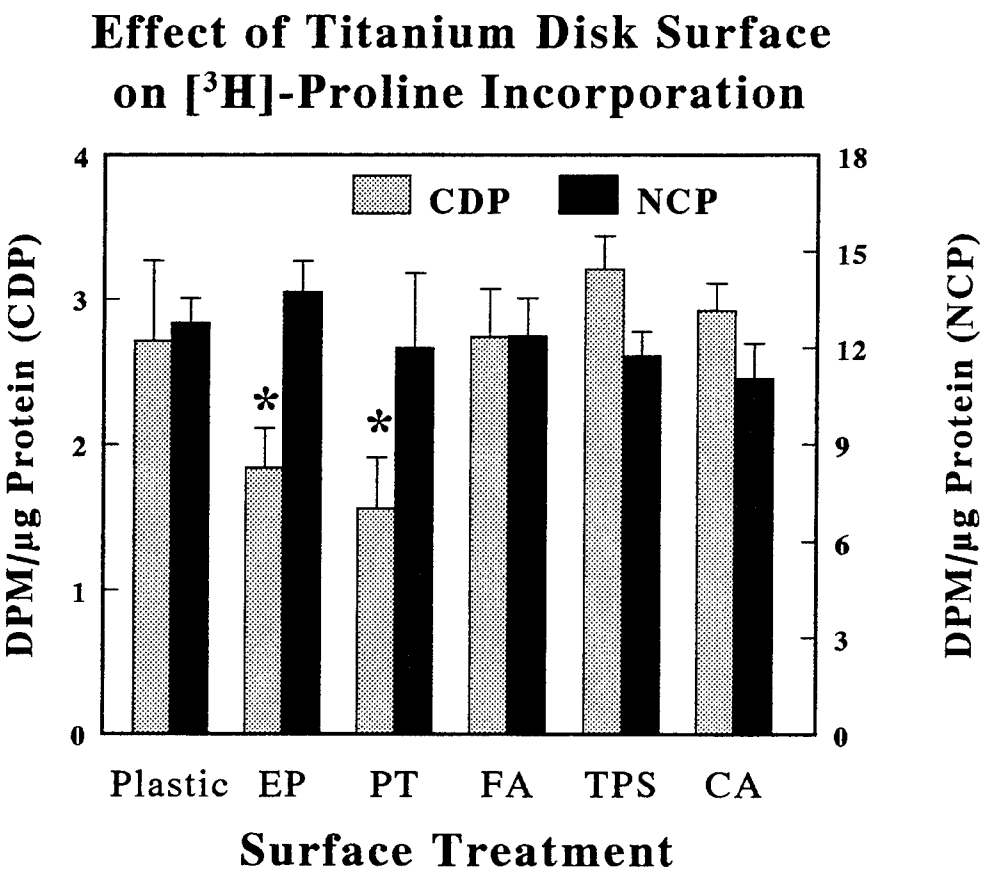

Effect of Titanium Disk Surface
on [3H]-Proline Incorporation
CDP
NCP
DPM/µg Protein (CDP)
DPM/µg Protein (NCP)
0
1
2
3
4
0
3
6
9
12
15
18
*
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 19.

EFFECT OF TITANIUM DISK SURFACE ON MG63 PERCENT COLLAGEN PRODUCTION

Values were derived from CDP and NCP production and are the mean ± standard error of the mean of six cultures.
*$p<0.05$, plastic v. EP, PT, or FA; $^{\#}$EP v. all other surfaces; $^{\bullet}$PT v. FA, TPS, or CA.

Data were derived from that shown in Figure 18.

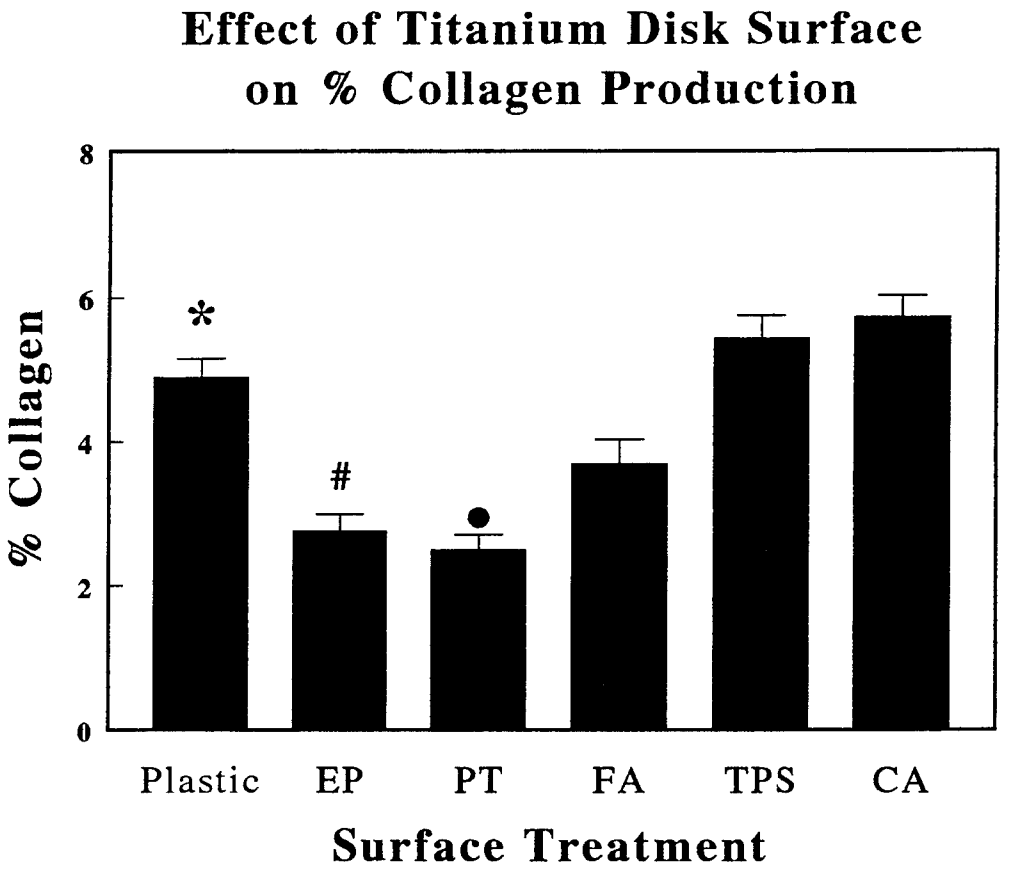

Effect of Titanium Disk Surface
on % Collagen Production
% Collagen
8
6
4
2
0
*
#
●
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 20.

EFFECT OF TITANIUM DISK SURFACE ON GC [$^3$H]-PROLINE INCORPORATION FOR CDP AND NCP PRODUCTION

Values shown are the mean ± standard error of the mean of six cultures.
*$p<0.05$, plastic v. all titanium surfaces.

Data are from one of two replicate experiments.

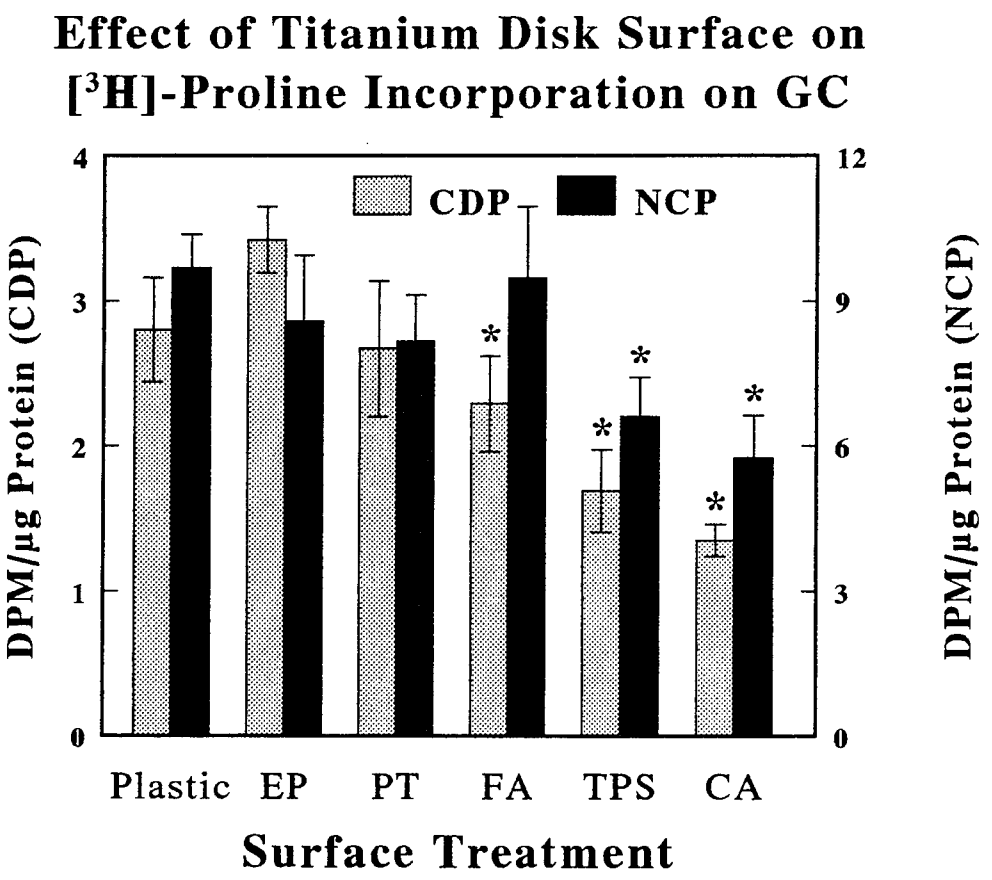

Effect of Titanium Disk Surface on
[3H]-Proline Incorporation on GC
CDP
NCP
DPM/µg Protein (CDP)
DPM/µg Protein (NCP)
0
1
2
3
4
0
3
6
9
12
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 21.

EFFECT OF TITANIUM DISK SURFACE ON RC [$^3$H]-PROLINE INCORPORATION FOR CDP AND NCP PRODUCTION

Values shown are the mean ± standard error of the mean of six cultures.
*$p<0.05$, plastic v. all titanium surfaces.

Data are from one of two replicate experiments.

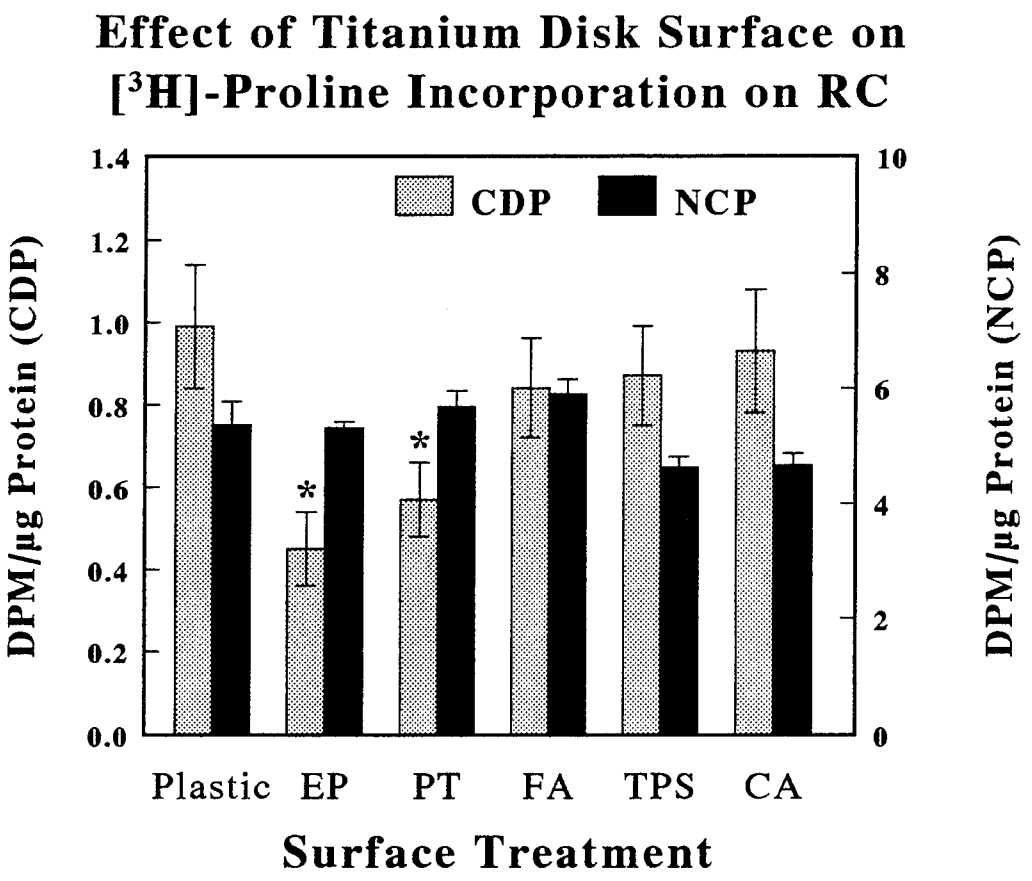
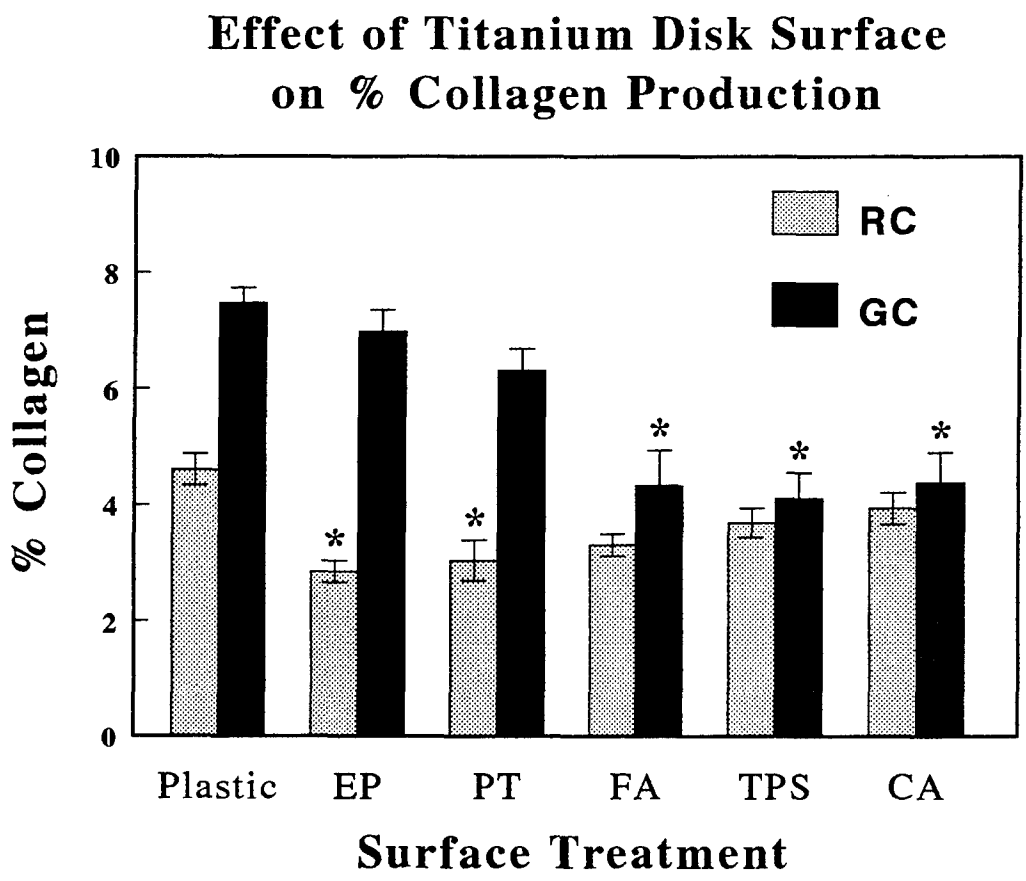

Effect of Titanium Disk Surface on
[3H]-Proline Incorporation on RC
DPM/µg Protein (CDP)
DPM/µg Protein (NCP)
CDP
NCP
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
8
6
4
2
0
*
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 22.

EFFECT OF TITANIUM DISK SURFACE ON GC AND RC PERCENT COLLAGEN PRODUCTION

Values were derived from CDP and NCP production and are the mean ± standard error of the mean of six cultures.
*$p<0.05$, plastic v. all titanium surfaces.

Data are derived from that shown in Figure 20 and Figure 21.


Effect of Titanium Disk Surface
on % Collagen Production
% Collagen
10
8
6
4
2
0
RC
GC
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

B. [$^{35}$S]-Sulfate incorporation

Results of [$^{35}$S]-sulfate incorporation appeared to be unaffected by cell type and surface texture. All surfaces and cell types revealed a significant inhibition in [$^{35}$S]-sulfate incorporation compared to the plastic control.

In MG63 cells, this reduction on the titanium surfaces compared to control can be seen in Figure 23. This effect was less pronounced in cells grown on FA-treated surfaces and more pronounced in cells grown on CA-treated surfaces. Cells cultured on CA incorporated significantly less [$^{35}$S]-sulfate than did those cultured on PT-, FA-, and TPS-treated surfaces.

Compared to plastic, [$^{35}$S]-sulfate incorporation was reduced on all surfaces with both GCs and RCs (Figure 24). In both cases, this effect was inversely related to surface roughness, although differences between titanium surfaces were not significant.

Figure 23.

EFFECT OF TITANIUM DISK SURFACE ON MG63 [$^{35}$S]-SULFATE INCORPORATION

Values shown are the mean ± standard error of the mean of six cultures.
*$p<0.05$: plastic v. titanium surfaces; $^{\#}$FA v. CA; $^{\bullet}$CA v. TPS or PT.

Data are from one of two replicate experiments.

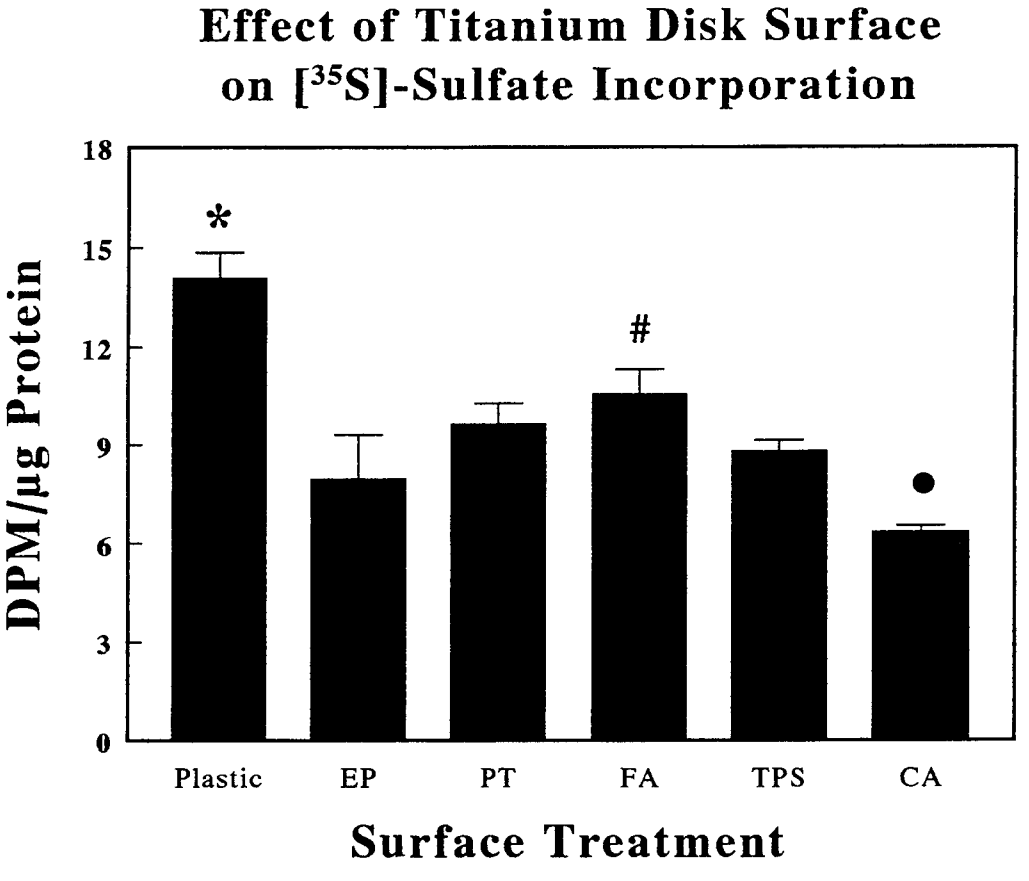
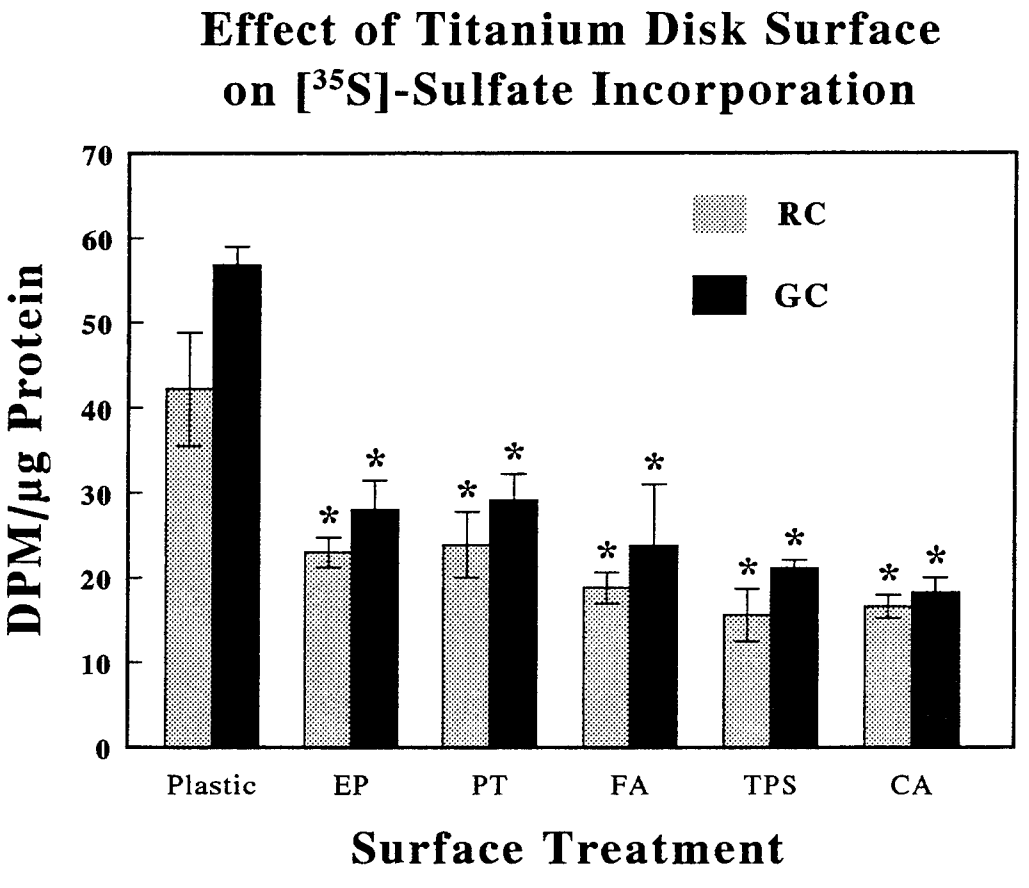

Effect of Titanium Disk Surface
on [35S]-Sulfate Incorporation
DPM/µg Protein
18
15
12
9
6
3
0
*
#
●
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

Figure 24.

EFFECT OF TITANIUM DISK SURFACE ON GC AND RC [$^{35}$S]-SULFATE INCORPORATION

Values shown are the mean ± standard error of the mean of six cultures.
*$p<0.05$, plastic v. all titanium surfaces.

Data are from one of two replicate experiments.


Effect of Titanium Disk Surface
on [35S]-Sulfate Incorporation
DPM/µg Protein
70
60
50
40
30
20
10
0
RC
GC
*
Plastic
EP
PT
FA
TPS
CA
Surface Treatment

6. Results of Re-sterilization and Re-use of Titanium Disks

A. Cell Proliferation

Cell number was sensitive to the surface treatment as was previously seen with MG63 cells (Figure 25). Twenty-four hours after confluence was achieved on plastic, the number of cells on titanium was similar to that found on plastic, except for TPS which contained significantly fewer cells. Except for TPS surfaces, which had an apparent reduction in cell number, no significant difference between new and used titanium disks was observed. A second trypsinization released additional cells from all surfaces, with the greatest number being released from TPS surfaces, however no differences were observed between the new and used surfaces.

[$^3$H]-Thymidine incorporation was significantly decreased on all titanium surfaces compared to plastic at 24 hours (Figure 26). The decrease in thymidine incorporation was directly related to surface roughness, with the greatest decrease found on TPS surfaces. No difference in [$^3$H]-Thymidine incorporation was found between new and used titanium disks except on CA surfaces, where [$^3$H]-Thymidine incorporation was decreased on used disks. Surface roughness and prior use produced a similar effect on [$^3$H]-Thymidine incorporation after forty-eight hours (Figure 27).

B. Cell Differentiation

Alkaline phosphatase activity in the cell layers varied with culture surface (Figure 28). Enzyme activity in cell layers after culture on FA and TPS surfaces was significantly lower than that seen on any of the other surfaces. There was no difference in alkaline phosphatase specific activity for culture on new or used surfaces.

Figure 25.

EFFECT OF TITANIUM DISK SURFACE ON MG63 CELL NUMBER
COMPARISON OF NEW VERSUS USED DISKS

Values are the mean ± standard error of the mean number of cells released from a particular titanium surface compared to plastic.
*$p<0.05$, titanium disk v. 1.
#$p<0.05$, new v. used disk.

Data are from one of two replicate experiments.

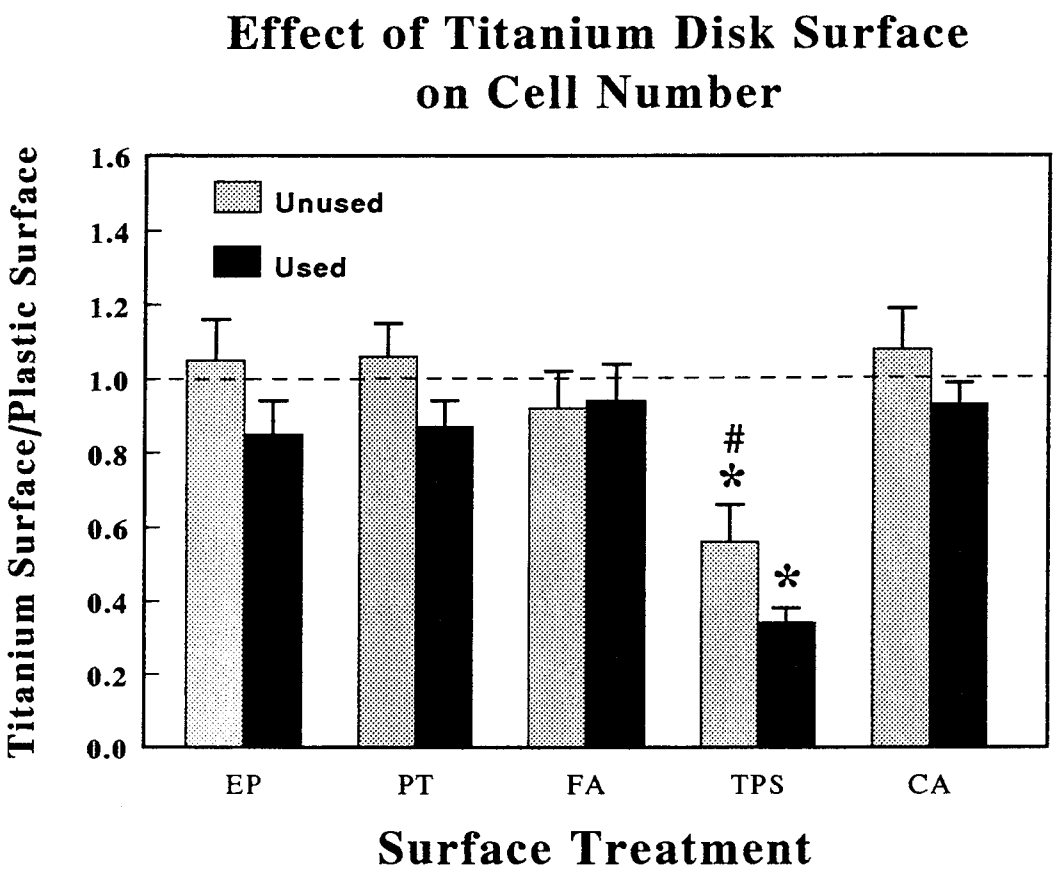

Effect of Titanium Disk Surface on Cell Number
Titanium Surface/Plastic Surface
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unused
Used
#
*
*
EP
PT
FA
TPS
CA
Surface Treatment

Figure 26.

EFFECT OF TITANIUM DISK SURFACE ON MG63 [$^3$H]-THYMIDINE INCORPORATION AFTER 24 HOURS: COMPARISON OF NEW VERSUS USED DISKS

Values are the mean ± standard error of the mean [$^3$H]-thymidine incorporation by cells cultured on a particular titanium surface compared to plastic.
*$p<0.05$, titanium disk v. 1
$^{\#}p<0.05$, new v. used disk.

Data are from one of two replicate experiments.

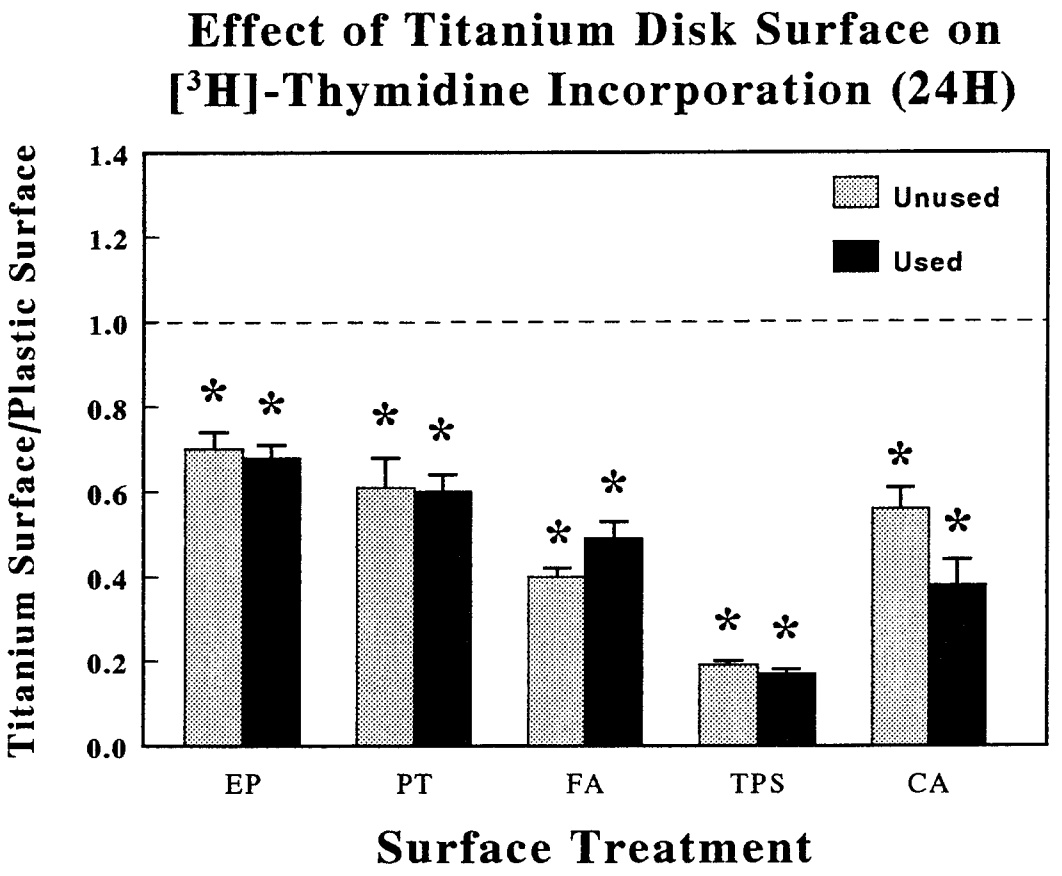

Effect of Titanium Disk Surface on
[3H]-Thymidine Incorporation (24H)
Titanium Surface/Plastic Surface
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unused
Used
*
EP
PT
FA
TPS
CA
Surface Treatment

Figure 27.

EFFECT OF TITANIUM DISK SURFACE ON MG63 [$^3$H]-THYMIDINE INCORPORATION AFTER 48 HOURS: COMPARISON OF NEW VERSUS USED DISKS

Values are the mean ± standard error of the mean [$^3$H]-thymidine incorporation by cells cultured on a particular titanium surface compared to plastic.
*$p<0.05$, titanium disk v. 1
$^{\#}p<0.05$, new v. used disk.

Data are from one of two replicate experiments.

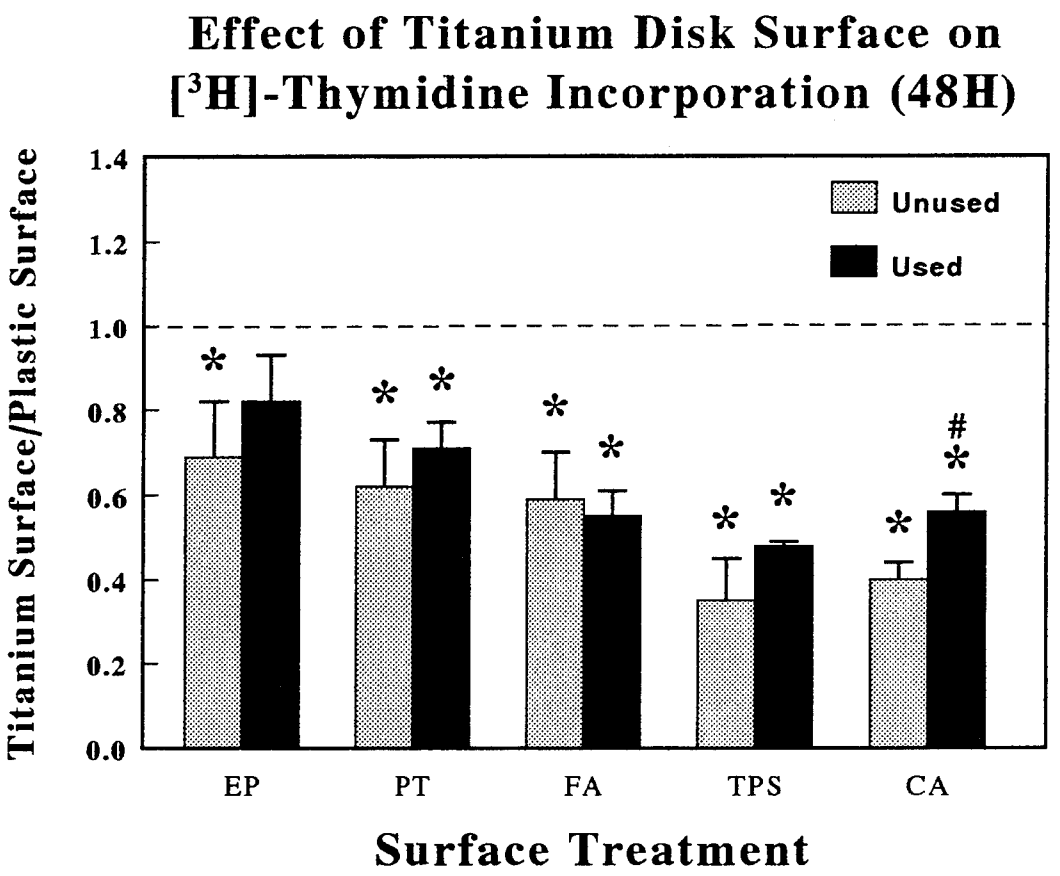
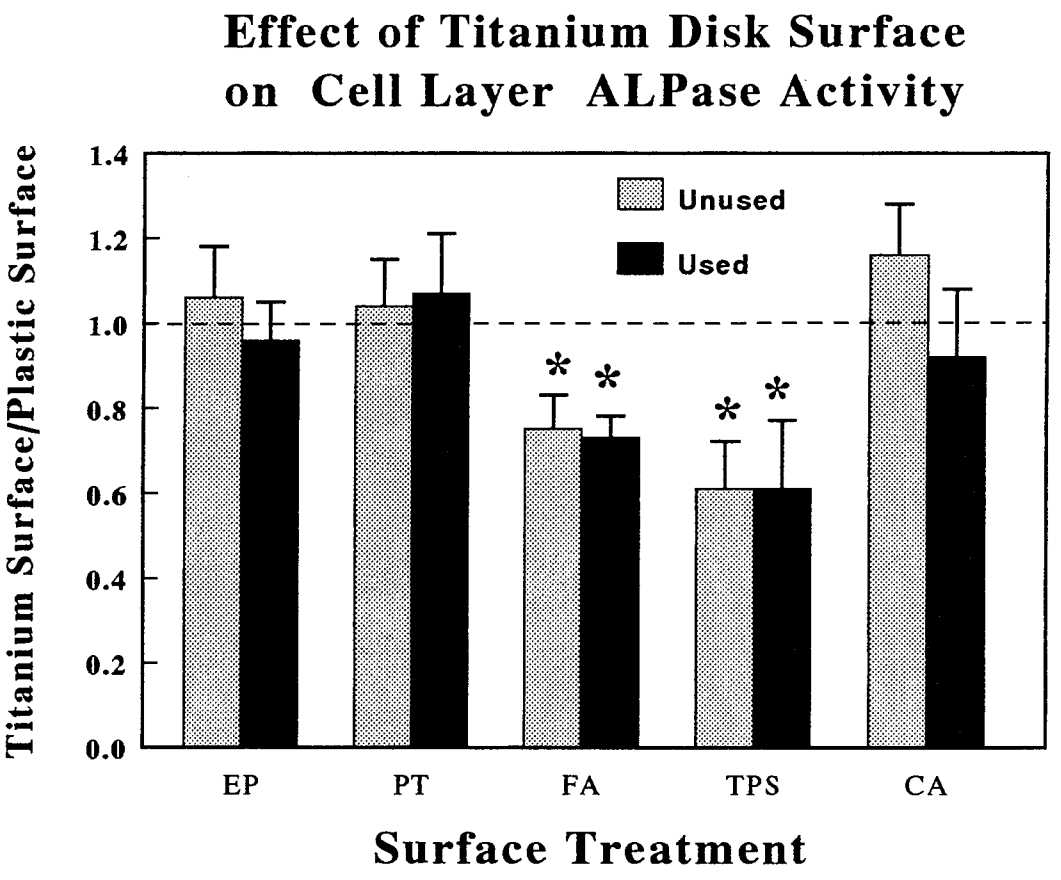

Effect of Titanium Disk Surface on
[3H]-Thymidine Incorporation (48H)
Titanium Surface/Plastic Surface
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unused
Used
*
*
*
*
*
*
*
*
*
#
*
EP
PT
FA
TPS
CA
Surface Treatment

Figure 28.

EFFECT OF TITANIUM DISK SURFACE ON MG63 CELL LAYER ALKALINE PHOSPHATASE ACTIVITY: COMPARISON OF NEW VERSUS USED DISKS

Cells were harvested by scraping the cell layer 24 hours after confluence on plastic.
Values are the mean ± standard error of the mean alkaline phosphatase specific activity of cultures grown on a particular titanium surface compared to plastic.
*$p<0.05$, titanium disk v. 1

Data are from one of two replicate experiments.


Effect of Titanium Disk Surface
on Cell Layer ALPase Activity
Titanium Surface/Plastic Surface
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unused
Used
*
*
*
*
EP
PT
FA
TPS
CA
Surface Treatment

When alkaline phosphatase specific activity was measured in isolated cells after culture for twenty-four hours post-confluence on TPS or CA, it was found to be lower than that found in cells cultured on any of the other surfaces (Figure 29). Whether or not the cells were cultured on new or used disks did not alter this response. In samples measured at 48 hours post-confluence, alkaline phosphatase specific activity was less in cells cultured on FA, TPS, and CA surfaces. (Figure 30). Again, this trend was the same whether or not new or used disks were employed.

C. Protein production

[$^3$H]-Uridine incorporation by cells cultured on plastic and CA-treated titanium was comparable (Figure 31), while that of cells cultured on EP-, PT, or FA-treated surfaces was significantly decreased. In contrast, [$^3$H]-Uridine incorporation on TPS surfaces was higher than that found on all other titanium surfaces. No difference in [$^3$H]-Uridine incorporation was observed between cells grown on new or used titanium disks.

Protein synthesis in the form of collagenase digestible and non-collagenase digestible proteins also affected culture surface as previously seen (Figure 32). CDP production by cells cultured on EP- and PT-treated surfaces was significantly reduced compared to all other surfaces, including plastic. CDP production on FA, TPS, and CA surfaces was comparable to that seen on plastic. No difference in CDP production was observed between new and used disks except on TPS, where significantly more CDP was produced on the new disks. NCP production by the cells was not affected by culturing on the different surfaces, nor was there a difference in NCP production between new and used disks (Figure 33). Percent collagen production was decreased by culture on EP-, PT-, and FA-treated surfaces compared with plastic and the other titanium surfaces (Figure 34). Production on PT was also less than on the other titanium surfaces

Figure 29.

EFFECT OF TITANIUM DISK SURFACE ON MG63 CELL ALKALINE PHOSPHATASE ACTIVITY AFTER 24 HOURS: COMPARISON OF NEW VERSUS USED DISKS

Cells were harvested by trypsinization 24 hours after confluence on plastic.
Values are the mean ± standard error of the mean of alkaline phosphatase specific activity of cells grown on a particular titanium surface compared to plastic.
*$p<0.05$, titanium disk v. 1

Data are from one of two replicate experiments.

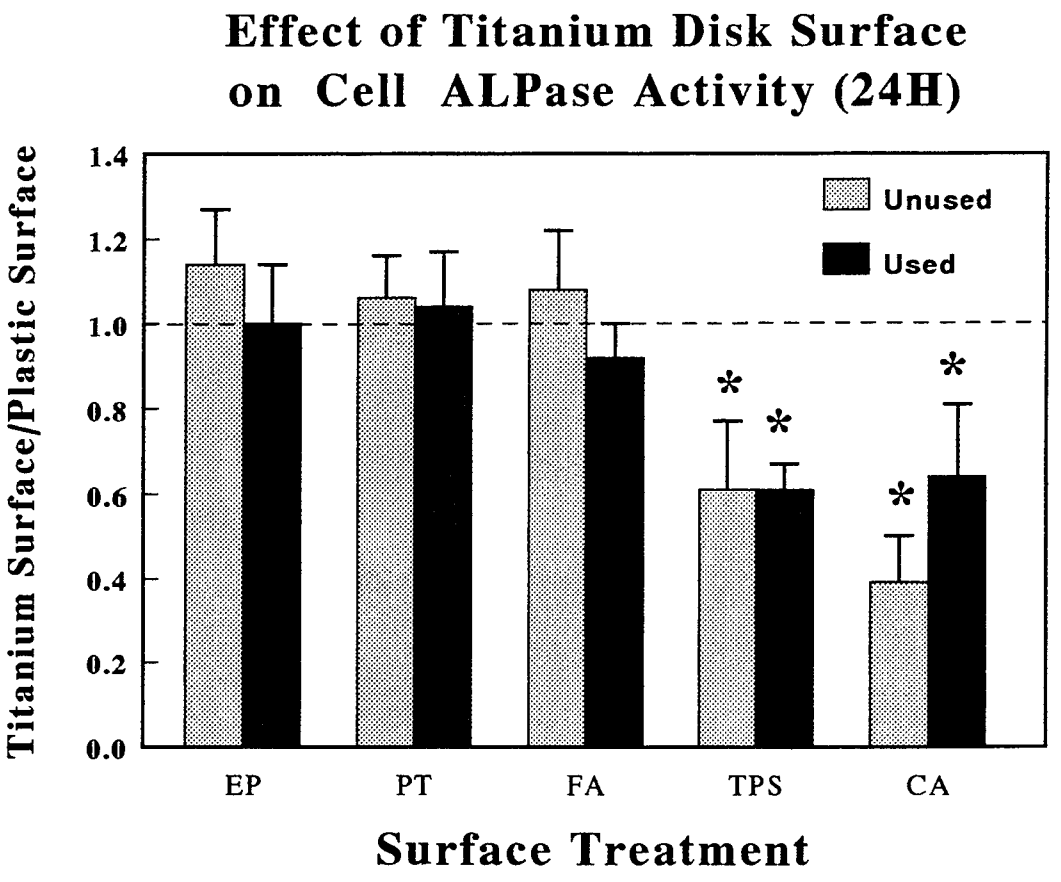

Effect of Titanium Disk Surface
on Cell ALPase Activity (24H)
Titanium Surface/Plastic Surface
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unused
Used
*
*
*
*
EP
PT
FA
TPS
CA
Surface Treatment

Figure 30.

EFFECT OF TITANIUM DISK SURFACE ON MG63 CELL ALKALINE PHOSPHATASE ACTIVITY AFTER 48 HOURS: COMPARISON OF NEW VERSUS USED DISKS

Cells were harvested by trypsinization 48 hours after confluence on plastic.
Values are the mean ± standard error of the mean of alkaline phosphatase specific activity of cells grown on a particular titanium surface compared to plastic.
*$p<0.05$, titanium disk v. 1

Data are from one of two replicate experiments.

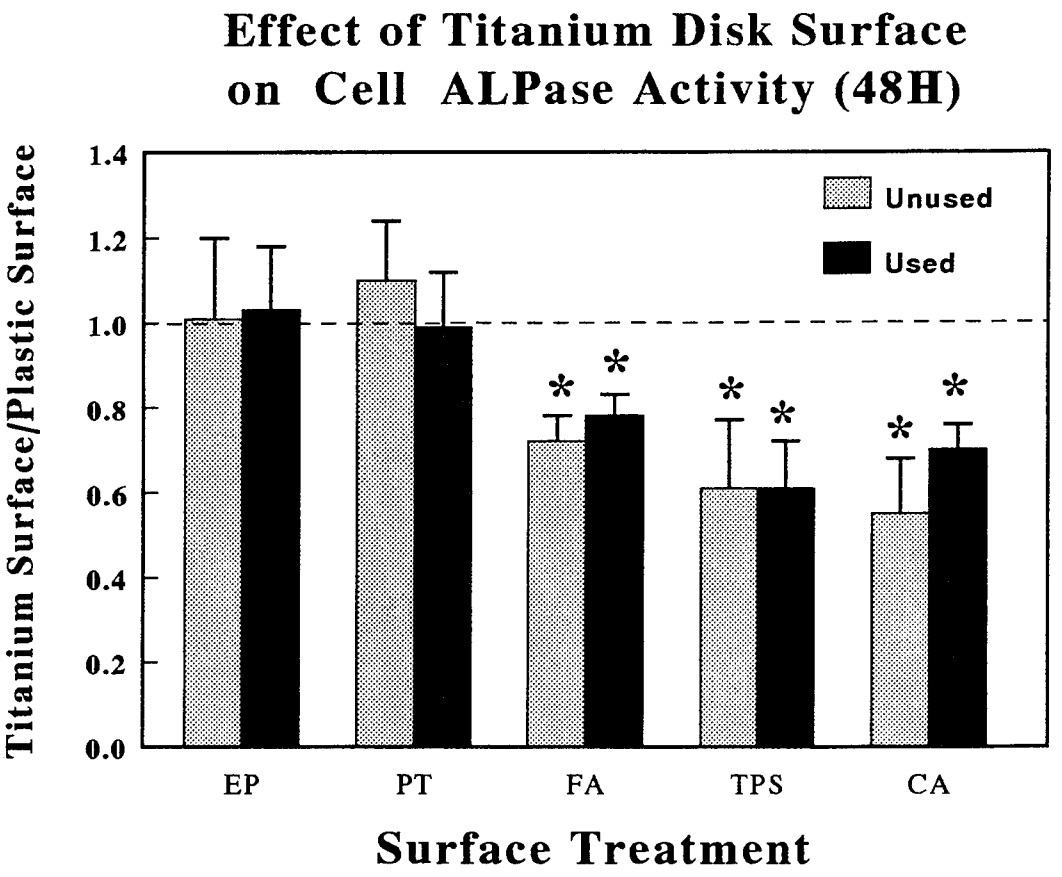

Effect of Titanium Disk Surface
on Cell ALPase Activity (48H)
Titanium Surface/Plastic Surface
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unused
Used
*
EP
PT
FA
TPS
CA
Surface Treatment

Figure 31.

EFFECT OF TITANIUM DISK SURFACE ON MG63 [$^3$H]-URIDINE INCORPORATION: COMPARISON OF NEW VERSUS USED DISKS

Values are the mean ± standard error of the mean [$^3$H]-uridine incorporation by cells grown on a particular titanium surface compared to plastic.
*$p<0.05$, titanium disk v. 1.

Data are from one of two replicate experiments.

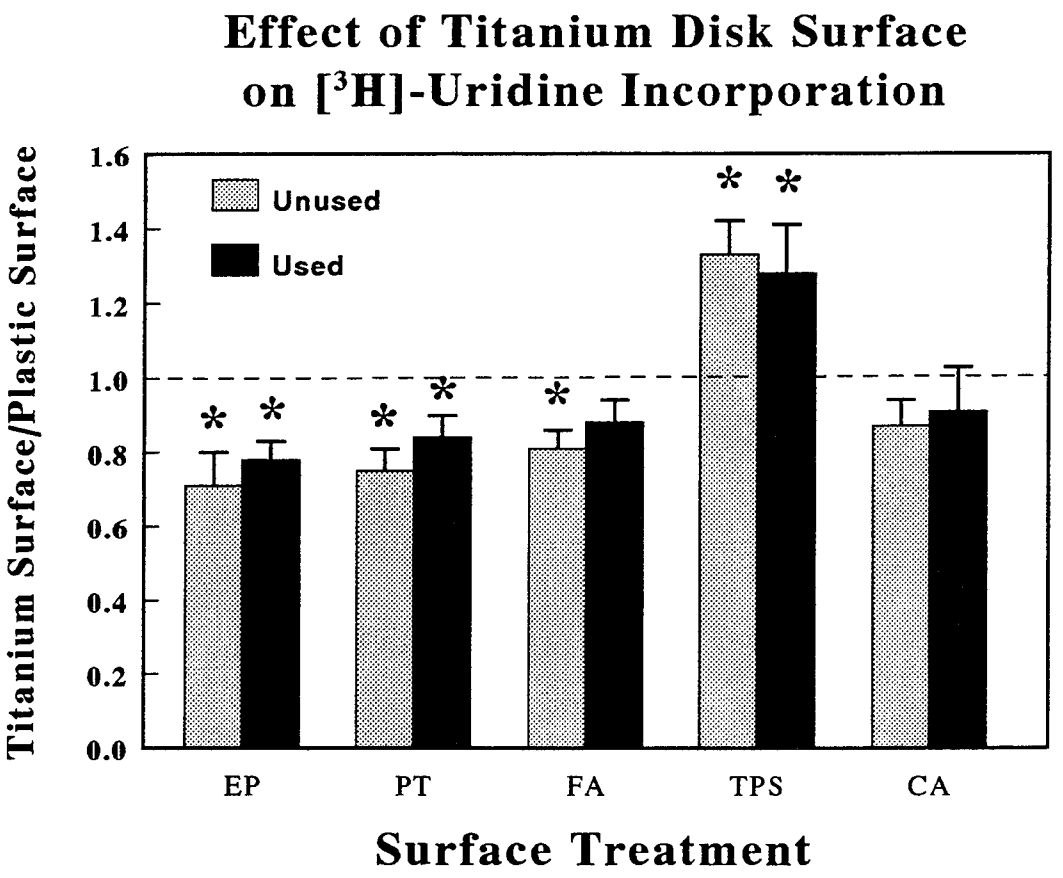

Effect of Titanium Disk Surface
on [3H]-Uridine Incorporation
Titanium Surface/Plastic Surface
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unused
Used
*
EP
PT
FA
TPS
CA
Surface Treatment

Figure 32.

EFFECT OF TITANIUM DISK SURFACE ON MG63 [$^3$H]-PROLINE INCORPORATION INTO CDP: COMPARISON OF NEW VERSUS USED DISKS

Values are the mean ± standard error of the mean of cells grown on a particular titanium surface compared to plastic.
*$p<0.05$, titanium disk v. 1
$^{\#}p<0.05$, new v. used disks.

Data are from one of two replicate experiments.

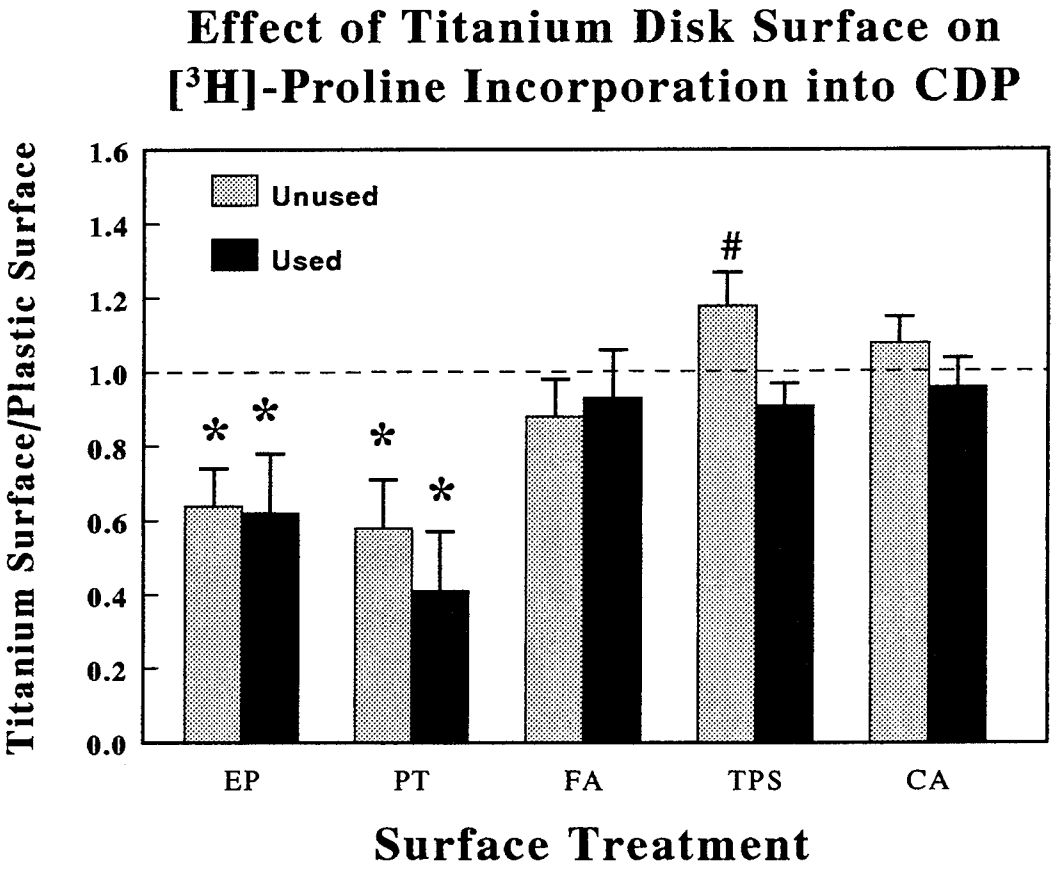

Effect of Titanium Disk Surface on
[3H]-Proline Incorporation into CDP
Titanium Surface/Plastic Surface
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unused
Used
*
*
*
*
#
EP
PT
FA
TPS
CA
Surface Treatment

Figure 33.

EFFECT OF TITANIUM DISK SURFACE ON MG63 [$^{3}$H]-PROLINE INCORPORATION INTO NCP: COMPARISON OF NEW VERSUS USED DISKS

Values are the mean ± standard error of the mean of cells grown on a particular titanium surface compared to plastic.

Data are from one of two replicate experiments.

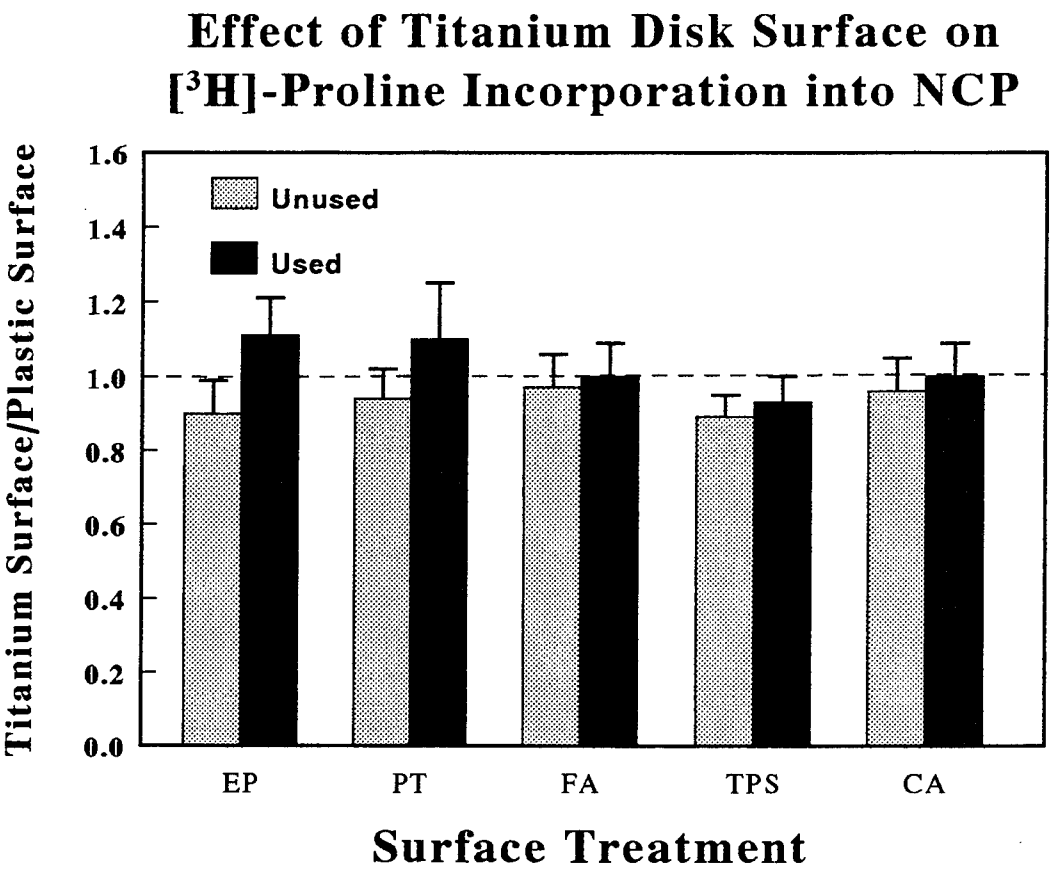
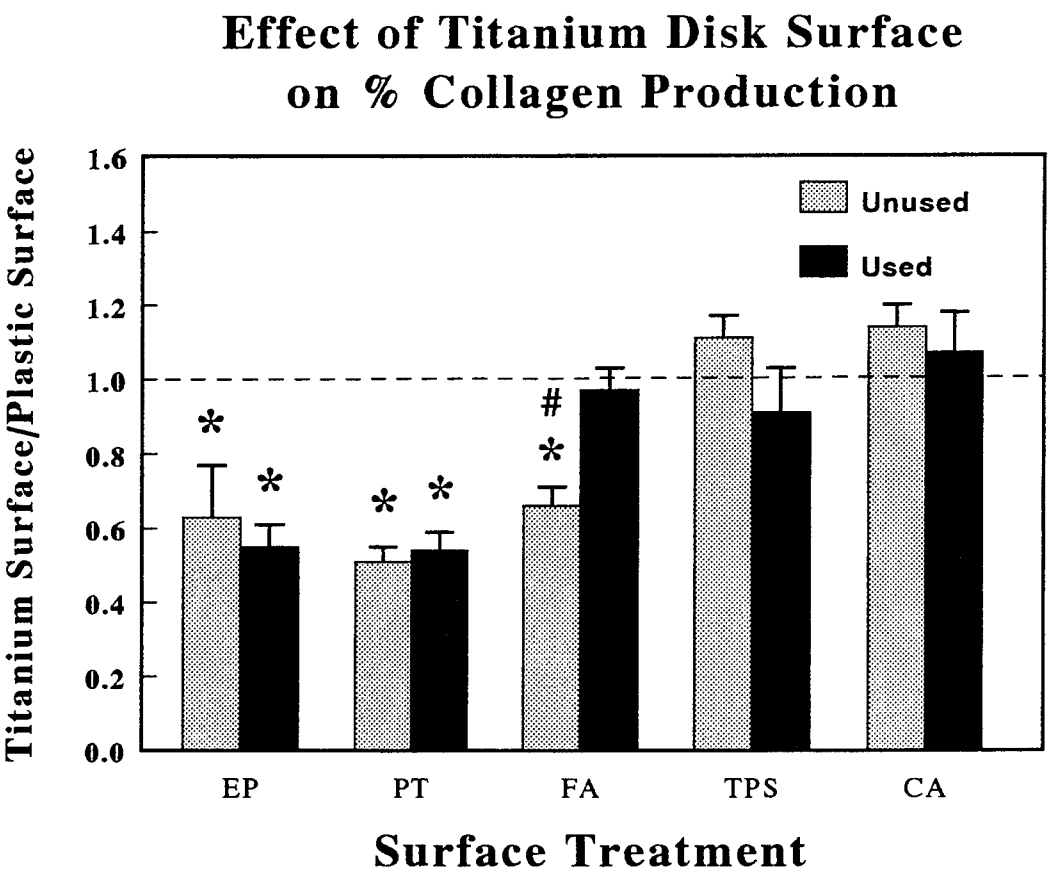

Effect of Titanium Disk Surface on
[3H]-Proline Incorporation into NCP
Titanium Surface/Plastic Surface
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unused
Used
EP
PT
FA
TPS
CA
Surface Treatment

Figure 34.

EFFECT OF TITANIUM DISK SURFACE ON MG63 PERCENT COLLAGEN PRODUCTION: COMPARISON OF NEW VERSUS USED DISKS

Values were derived from CDP and NCP production and are the mean ± standard error of the mean percent collagen produced by cells grown on a particular titanium surface compared to plastic.
*$p<0.05$, titanium disk v. 1
#$p<0.05$, new v. used disks.

Data were derived from that shown in Figures 32 and 33.


Effect of Titanium Disk Surface
on % Collagen Production
Titanium Surface/Plastic Surface
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unused
Used
*
*
*
*
#
*
EP
PT
FA
TPS
CA
Surface Treatment

A similar effect on percent collagen production was also found on new and used surfaces except for FA surfaces. MG63 cells cultured on used FA disks produced more collagen than those cells cultured on the new surfaces. Moreover, a similar percent collagen production was found on used FA and plastic surfaces (Figure 34).

D. Matrix production

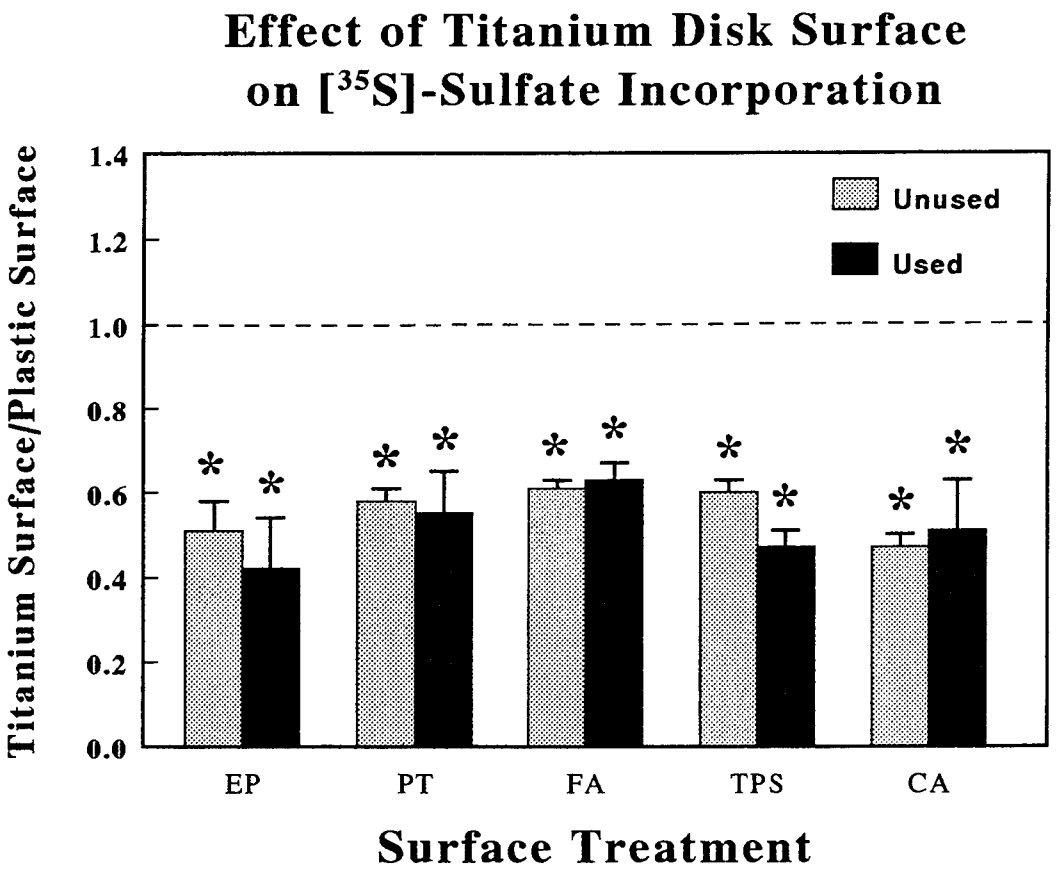
[$^{35}$S]-Sulfate incorporation was uniformly reduced on all titanium surfaces when compared to plastic (Figure 35). This effect was less pronounced in cells grown on FA-treated surfaces and more pronounced in cells grown on CA and EP surfaces. Cells grown on new and used surfaces exhibited comparable [$^{35}$S]-Sulfate incorporation.

Figure 35.

EFFECT OF TITANIUM DISK SURFACE ON MG63 [$^{35}$S]-SULFATE INCORPORATION: COMPARISON OF NEW VERSUS USED DISKS

Values are the mean ± standard error of the mean [$^{35}$S]-Sulfate incorporation by cells grown on a particular titanium surface compared to plastic.
*$p<0.05$, titanium disk v. 1.

Data are from one of two replicate experiments.


Effect of Titanium Disk Surface
on [35S]-Sulfate Incorporation
Titanium Surface/Plastic Surface
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Unused
Used
EP
PT
FA
TPS
CA
Surface Treatment

# IV. DISCUSSION

1. Surface Effects

This study examined the effects of various factors which may influence biologic responses of cells to titanium surfaces. The results of the surface characterization has shown that different surface treatments resulted in significantly different surface textures and morphologies. In the case of EP-treated and PT-treated disks, although the two disk types were viusally indistiguishable, electron microscopy and profile evaluation demonstrated the distinct natures of each. This is in agreement with the findings of Wennerberg *et al* (1993) on their evaluation of thirteen commercially available implant systems. They also noted the wide variation in microtopography of the implant surfaces. They also stated that there was even significant variation within implant systems. This is understandable based on the evaluations performed in this study. The surface treatment regimens cannot produce an exact duplicate of the surface from sample to sample due to the nature of the surface treatment procedures. Thus, in any implant, although the overall texture can be modified, variations within that surface will exist.

The presence of the oxide layer was confirmed through our examinations, as was the nature and thickness of this layer. Results of the Auger electron spectroscopy revealed a Ti:O ratio ranging from 0.2 to 0.4. The most common, oxygen-rich, stable compound of titanium is $TiO_2$, which has a Ti:O ratio of 0.5. Although our findings confirm the oxygen rich nature of the titanium compounds on the surface, the ratio implies the presence of a compound other than $TiO_2$. This is in agreement with Olefjord and Hansson (1993) who evaluated four different implant systems using electron spectroscopy and found that although $TiO_2$ was the most

common covering oxide layer, divalent and trivalent states of titanium were also present, and represented the presence of both TiO and $Ti_2O_3$.

It was interesting to note that the oxide layer increased with increasing surface roughness, and this thickness seemed to be unaffected by steam sterilization. The differences in oxide layer among the treated surfaces can alter cell behavior. By changing the surface energy, a factor that can change implant acceptance *in vivo* (Kasemo and Lausmaa, 1986; Baier *et al*, 1984, Chehroudi *et al*, 1991), the adsorption of serum components may be altered. Subsequent changes in the thickness of TiO2 may also affect titanium leaching, ultimately affecting the cells. The thickest oxide layers being found on the roughest surfaces may also alter cell activity in that increased surface area may increase ion release. Our findings on the stability of the oxide layer after sterilization agrees with Machnee *et al* (1993), who found no change in surface oxide layer thickness after four different surface treatments.

The dominant elements found on the titanium surfaces were titanium, oxygen and carbon. Trace amounts of phosphorous, calcium, potassium, chloride and sodium were also noted. Surface contaminants mentioned in other studies include fluorine, calcium and zinc (Olefjord and Hansson, 1993), oxygen, carbon and sodium, as well as nitrogen, magnesium, silicone, chloride, manganese, tin, silver and arsenic (Klauber *et al*, 1990). It appears that there is general agreement in the presence and types of surface contaminants. Also, no implant seems to be without surface contamination despite attempts at developing and maintaining a scrupulously clean surface. In assessing sources of contamination, basically any substance which comes into contact with the implant material, as well as the metal itself, is a potential source of contamination. Clinically, the possibility of eliminating or complete prevention of contamination is probably unrealistic. In terms of clinical performance, the study by Stanford *et al* (1994) evaluating use of an autoclave with a standard water source versus autoclaving with ultrapure water found no difference in osteocalcin production by cultured bone cells. Thus, the implication is that clinical effects may be minimal, or not readily detectable at this time.

2. Effect of Surface Roughness

Most recent studies of surface roughness have focused on cell attachment (Michaels *et al*, 1989; Bowers *et al*, 1992) and have shown better cell attachment on rough surfaces. The effect of surface texture on later time frames of cell differentiation, protein synthesis, matrix production, and calcification is as important as cell attachment and will have a major role in implant prognosis and success. We examined the effect of surface roughness at a later time point; when the cells were at confluency (at least on the plastic control surfaces). The results show that surface roughness has a major effect on cells and that many aspects of cellular activity, including cell morphology, are affected in a surface-specific manner.

Cell proliferation was difficult to assess in cultures grown on rough-surfaced titanium disks. Scanning electron microscopy and laser scanning microscopy verified that the cultured cells had migrated into the pits of the disk and thus were more resistant to release by trypsinization. While TPS disks had the lowest cell numbers after the first trypsinization, the greatest number of cells was released from the TPS surfaces upon a second trypsinization. This strongly suggests a direct correlation between surface roughness and the number of cells trapped within the 3-dimensional surface of the disk. Further, the total number of cells released from the rough surfaces, including those trapped within the surface and released after the second trypsinization, showed that surface roughness and topography inhibit cell replication (ie: decreased cell number) and possibly cell adhesion in both RCs and MG63 cells. This was in contrast to results seen with GCs. Although the first trypsinization showed a decrease in cell number from the rough surfaces compared to plastic, the second trypsinization released significantly more cells from the rough surfaces. The total number of GCs released from the different surfaces were comparable, implying that GC cell number is probably insensitive to surface roughness.

Similarly, the nature of the surface also appeared to affect the rate of proliferation, with cell specific results. At the earliest time point examined, all cells appeared to proliferate readily on both EP- and PT-treated surfaces and plastic. The proliferation rate was negatively impacted by

surfac roughness in RC and MG63 cells, since increasing surface roughness resulted in decreased levels of [$^3$H]-thymidine incorporation. Again, in contrast, GC proliferation was enhanced on rough surfaces.

Cell differentiation, assessed as a function of alkaline phosphatase specific activity, was affected by surface roughness. Enzyme activity of MG63 cells or cell layers was significantly decreased after culture on FA and TPS surfaces, while no inhibition was observed in samples obtained from smooth surfaces. In the CA group, however, the results suggest that the inhibition of alkaline phosphatase specific activity associated with surface roughness occurred at the cellular level, since changes in enzyme activity in the cell layer did not parallel those seen in the isolated cells. In fact, matrix activity in CA cultures must have been quite high, since the inhibition seen in the cells was completely obscured by the activity present in the matrix. Since earlier studies (Boyan *et al*, 1989) suggest that alkaline phosphatase in the cell layer of MG63 cells is largely located in matrix vesicles (organelles which play a role in calcification) these results indicate that matrix vesicle alkaline phosphatase specific activity, and possibly calcification, is enhanced by surface roughness.

Alkaline phosphatase specific activity in the cell layer of GCs after culture on CA disks remained elevated, while enzyme content of the cells decreased. This indicates that the enzyme was especially localized in the matrix of GCs after culture on CA disks. In contrast, enzyme in the matrix of RCs after culture on CA was the same as that found with FA and TPS, which were significantly decreased compared to the smooth surfaces and the plastic control.

Protein synthesis and matrix production were also surface-sensitive, and cell-sensitive. Protein synthesis and matrix production in MG63 cells displayed inhibition on the smoothest surfaces, particularly those treated with EP. In contrast, RNA synthesis was enhanced in cell cultures on the roughest surface, TPS. [$^3$H]-Uridine incorporation reflected changes in CDP synthesis, suggesting that collagen production may have been selectively depressed in cells cultured on EP. While this effect was evident in cultures twenty-four hours post-confluence, it

was no longer evident by forty-eight hours. Other studies have shown similar effects of titanium on matrix production (Itakura *et al*, 1988; Goldring *et al*, 1990). These results paralleled the results seen with the RCs. In contrast, results of surface roughness on matrix production on GCs showed an inhibition of NCP and CDP production on rough surfaces.

The behavior of MG63 cells on the CA surface was unique. Although it had one of the roughest surfaces, it had a very regular surface geometry. Osteoblast cell number and proliferation and cell layer alkaline phosphatase were similar to that seen on smoother materials. In contrast, cellular alkaline phosphatase activity and matrix production were similar to that of cells grown on the rougher surfaces. These results indicate that CA-treated surfaces enhanced both matrix production and calcification. They may also indicate that surface regularity is an important factor in cell differentiation and calcification. These results show that not only roughness, but many facets of surface topography, are important in the biological performance of different materials.

*In vivo* studies (Thomas and Cook, 1985; Wilke *et al*, 1990) have shown that bone is induced with rough-surfaced titanium, whereas smooth surfaces are almost entirely covered by fibrous tissue. The advantage of rough surfaces can be explained by the results of this study, which showed better matrix production on rough surfaces.

The effect of surface roughness on cells can be the result of the surface roughness itself or the result of the reaction which occurs as the material surface is conditioned by the media and serum (Hench and Paschall, 1973; Jarcho *et al*, 1988). This initial interaction produces a layer of macromolecules that modify the behavior of the cells. Fibronectin, a cell adhesion protein present in serum, has been shown to mediate cell attachment and spreading on artificial substrates by interacting with glycosaminoglycans and the cytoskeleton (Grinell, 1978; Doillon *et al*, 1987). It is possible that the rough surfaces may have adsorbed more fibronectin than the other surfaces (Weiss and Reddi, 1981; Pearson et al, 1988), thus enhancing the synthesis of extracellular matrix proteins.

3. Effect of Cell Variations

The above discussion has alluded to some of the cell-related differences seen in the results. Cell type, and maybe more importantly, cell maturation produces variations in results to titanium surfaces. This study's results for RCs and GCs show for the first time that the effect of surface roughness is maturation-dependent and that cells at different stages of differentiation respond differently to the same surface. This is in agreement with prior studies showing that hormones and local factors, such as vitamin D and TGF-β, affect chondrocytes differently at different stages of maturation (Schwartz *et al*, 1992a, 1992b; Schwartz *et al*, 1993; Sylvia *et al*, 1993). Gerstenfeld *et al* (1990) showed that, during differentiation, various markers of phenotypic maturation change with differentiation.

Although all three cell types produced different results to the biochemical assays, the results for RCs and MG63 cells were quite similar. Both cells showed an inhibition of cell number and proliferation on rough surfaces, as well as an inhibition of alkaline phosphatase specific activity and proteoglycan synthesis. In addition, both RCs and MG63 cells showed reduction of collagen production only on smooth surfaces. When MG63 cells were first characterized (Franchesi *et al*, 1985), it was found that this cell line behaved as expected for a pre-osteoblast, which means that they were immature osteoblasts. The response of MG63 cells to a surface, which is similar to the response of RCs (also a less mature cell), further supports the results showing that cell maturation affects cell response to a surface.

This has important implications when evaluating studies using cell models to determine an effect. Although it is well known that different cell types produce varying results, the stage of cell maturation is rarely evaluated or even mentioned. This factor should be taken into account when developing a study model, and when evaluating results of cell response. This highlights the difficulty in assessing results in which a cell explant model is used, since the cells are usually at various stages of maturation.

4. Sterilization and Re-use Results

The results of this study show, for the first time, that titanium disks exposed to serum and cell growth for more than a week can be cleaned, re-sterilized and, when used again, will affect cell growth in a manner similar to that seen on the original disk. There are some differences, however, particularly on the rougher surfaces. Cell number was reduced on used TPS surfaces and [$^3$H]-thymidine incorporation was reduced on used CA surfaces. It is likely that these small, but significant, reductions were a consequence of the difficulty in thoroughly removing all organic debris from these surfaces.

In general, the effects of surface roughness and topography on cell differentiation and matrix synthesis, noted earlier, were unaffected by re-use of these disks. This suggests that the surface characterisitics eliciting cellular response were unaltered by the cleaning and sterilization protocols as well as by cellular conditioning over the one-week culture period. The single exception to this was collagen production. [$^3$H]-proline incorporation into CDP was greater on new TPS disks than on used ones, and percent collagen synthesis (derived mathematically from the CDP and NCP data) was less on new FA disks. These differences only reflect protein synthesis during the labeling period and do not discriminate among protein species or collagen types. Thus, the presence or absence of changes in CDP and NCP production on new or used disks can only serve as first approximations of the effects of surface treatment on cell response. The limited differences observed in alkaline phosphatase specific activity and matrix synthesis, including [$^{35}$S]-sulfate incorporation, suggest that any alterations are subtle and may not have been detectable using these assays.

Any treatment of a material surface, either *in vivo* or *in vitro*, has the potential to change its surface characteristics to some extent (Cox and Smith, 1985; Smith *et al*, 1989; Smith *et al*, 1991), compromising its biocompatibility and, eventually, success of the procedure. Although it is clear that a scrupulously clean surface is important (Baier and Meyer,1988), regular autoclave sterilization changes the surface characteristics of a material (Doundoulakis, 1987; Lausmaa *et al*,

1985; Keller, 1990), which can lead to implant failure. The results of this study indicate that with simple cleaning and a single re-sterilization, even rough surfaces can be used without changing general cell behavior, which is very sensitive to environmental conditions (Windeler *et al*, 1991; Hambleton *et al*, 1994).

One factor which may have contributed to the similarity in cell response to the new and used surfaces is the fact that many of the physical properties of titanium surfaces were essentially unaltered by sterilization (as seen in this study). This confirms the observations of Machnee *et al* (1993), where surface oxide layer thickness did not change after sterilization. It is not known whether cell culture altered the surface properties of the titanium disks, but Aparicio and Olive (1992) noted no change in the surface oxide layer or surface roughness of clinically failed implants.

The results suggest the possibility that implants may be re-used, especially in the same patient, if they receive the appropriate treatment. Rougher titanium surfaces may require more extensive cleaning procedures, possibly something along the lines of plasma cleaning which has been shown to be effective in removing organic contaminants from implant surfaces. These results may also explain why bone regeneration can occur around an implant which has a bone defect, since the implant itself seems to be unaffected by cellular conditioning.

5. Significance

In summary, the results of this study have shown that the MG63 and the chondrocyte cell models can be effectively used to assess the effect of varying titanium surface roughness on cell metabolism. Although much is known about effects of factors influencing implant success, much more research is needed. Optimization of factors influencing incorporation of the implant into the host body could increase the chances for successful implantation. Determination of factors improving implant integration would benefit both the medical and dental communities. This study has shown the need for *in vitro* studies to provide basic information for further studies (ie: effect

of surface on cells; effect of cells, and stage of maturation on results), and to shed light on reasons for conflicting findings in other studies.

6. Future Studies

The results of this study support the possibility of purposely altering implant surface to produce desired effects. This may be pursued through fabrication of surface textures, microgeometry, and anatomic forms which may select for specific cell populations to appose the implant. Surface treatments may also be controlled in order to modify biologic response. Besides optimization of sterilization methods, the possibility exists for alteration of the implant surface to allow preferential adsorption of matrix proteins which may alter cell attachment. Also, the addition of implant coatings with materials such as specific growth factors may allow selection of a desired cell to populate the implant surface, or enhance healing and integration around the implant.

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# VITA

Janet Yun-Chieh Martin [redacted] She immigrated to the United States in 1968, and became a United States citizen in 1976. After graduating from Lahser High School in 1980, she began undergraduate studies at the University of Detroit. Instead of continuing with a Bachelor of Science degree in Chemistry, she opted instead to enter the University of Detroit Dental School in 1983. She graduated in May of 1987, and received the Doctor of Dental Surgery degree. She was also inducted into Omicron Kappa Upsilon, the dental honors society, at graduation.

Immediately after graduation from dental school, she received a commission in the United States Air Force and entered a one year general practice residency at Offutt Air Force Base in Omaha, Nebraska. Upon completion of the GPR, she was sent to Hickam Air Force Base in Hawaii and spent 5 years practicing general dentistry. She married Michael William Martin, also a dentist in the Air Force, while stationed in Hawaii.

In July of 1993, she entered the Post-Doctoral Periodontics Program at the University of Texas Health Science Center at San Antonio in conjunction with Wilford Hall Medical Center. She was admitted to candidacy for the Master of Science degree at the Graduate School of Biomedical Sciences in 1994.